Functional characterization of the nucleoporin CAN and CAN-derived leukemia-specific fusion proteins

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Functionele karakterisatie van de nucleoporine CAN en CAN-afgeleide leukemie-specifieke fusie-eiwitten

Proefschrift

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"Though we travel the world over to find the beautiful,
we must carry it with us or we find it not."

Ralph Waldo Emerson

In herinnering aan mijn vader
Voor moeder

Contents

Outline

Chromosome translocations are cytogenetically visible genetic abnormalities that are often associated with specific tumors. Characterization of the genes at the chromosome breakpoints can give insights into the processes that transform normal cells to tumor cells. The (6;9) translocation, associated with acute myeloid leukemia and myelodysplastic syndrome, fuses the *DEK* gene to *CAN* and results in the expression of a chimeric *DEK-CAN* gene. In a second chromosome rearrangement, found in one patient with acute undifferentiated leukemia, *CAN* is fused to *SET*. Knowledge of the normal functions of the proteins encoded by the fusion partners is indispensable in understanding the mechanisms by which DEK-CAN and SET-CAN contribute to leukemogenesis. The aim of the research described here is to define the functions of CAN, which was identified as a nuclear pore complex component (nucleoporin), and of the CAN-derived fusion proteins.

This thesis starts out with an introduction about chromosome aberrations in hematopoietic malignancies, that result in the generation of fusion genes. The DEK, SET, and CAN proteins are introduced, and a number of molecular mechanisms in oncogenic transformation by fusion proteins are highlighted with examples (Chapter 1). The chapters that follow describe our experimental work concerning the functions of CAN and DEK-CAN. First, we studied the consequences of loss of CAN function after disruption of the mouse *CAN* gene by homologous recombination (Chapter 2). Second, the effects of CAN and DEK-CAN expression on the growth, differentiation and survival of myeloid precursor cells were investigated (Chapter 3). Third, we studied the localization of the CAN protein within the nuclear pore complex using immunoelectron microscopy (Chapter 4). An additional approach to unravel the function of CAN was to identify CAN-interacting proteins by coimmunoprecipitation (Chapter 5). Lastly, sensitive molecular detection of the (6;9) translocation was applied towards the diagnosis and follow-up of an acute myeloid leukemia patient (Chapter 6). CAN emerges from these studies as an essential factor involved in both nuclear protein import and mRNA export through the nuclear pore. These findings have implications for the possible molecular mechanism of leukemogenic transformation by the CAN-derived fusion proteins (Chapter 7).

1 Fusion proteins in leukemia

1 Introduction

Cancer is the result of the accumulation of multiple genetic changes. Some of these alterations, that can either be initiating or progression-associated events, involve gross chromosomal abnormalities, that can be either structural or numerical. Structural alterations include translocations, inversions, deletions, insertions, and amplifications, whereas numerical abnormalities result in losses or duplications of whole chromosomes. Genes implicated in cancer can be subdivided into two classes, the proto-oncogenes and the tumor suppressor genes. Proto-oncogenes are activated by dominant gain of function mutations, whereas tumor suppressor genes contribute to oncogenicity through loss of function.

For proper growth and differentiation cells depend on regulatory signals from their environment. The cell membrane contains receptors that enable cells to respond to specific soluble factors or extracellular matrix proteins. Upon ligand binding, intracellular second messenger molecules are activated, often resulting in the phosphorylation of target proteins. Some ligands, such as steroid hormones, do not need a membrane receptor but pass the lipid bilayer directly and bind to intracellular receptors. Molecules transmitting the signal to the nucleus, enter via an active import mechanism through the nuclear pore complex. Inside the nucleus, these signals eventually lead to activation of transcription factors that bind to specific DNA sequences. These transcription factors affect the expression of responder genes that are crucial for the regulation of cell proliferation or differentiation. Changes in any of the genes encoding the proteins taking part in these cascades can deregulate proper growth and differentiation and contribute to the transformation of a normal cell to a cancer cell.

2 Chromosome aberrations in hematopoietic malignancies

The production of blood cells is normally regulated by a homeostatic mechanism. Acute leukemia is the uncontrolled proliferation or expansion of hematopoietic cells that do not retain the capacity to differentiate normally to mature blood cells. Some chronic hematologic disorders display only part of the full leukemic phenotype - either growth expansion (myeloproliferative syndromes) or a differentiation block (myelodysplasia) - yet both conditions can progress to acute leukemia. These observations suggest that full leukemic transformation requires multiple genetic changes, resulting in defects in both growth and differentiation (Sawyers et al., 1991).

A characteristic of many leukemias is the presence of recurring and highly consistent chromosome rearrangements, such as translocations and inversions, that usually generate chimeric genes which are considered to be involved in tumorigenesis. Sometimes the chromosome breakpoints occur in the regulatory sequences of two genes. This can lead to activation of a proto-oncogene when it comes to lie near a promoter or enhancer of another gene, such as those of the T-cell receptor or immunoglobulin genes. Aberrant expression of the translocated proto-oncogene is often associated with lymphomas or lymphocytic leukemia of T- or B-cells. Mostly however, the chromosome breaks are such that a gene fusion is created that encodes a chimeric protein with protein segments of both genes at the translocation breakpoints. Fusion genes encoding tumor-specific proteins are associated with different types of leukemias and lymphomas, as well as with solid tumors (Rabbitts, 1994).

3 Oncogenic fusion proteins

Chimeric genes encoding fusion proteins are generated when breakpoints of chromosomal translocations or inversions are located in introns flanking codogenic exons of two fusion partners. The breaks generally occur within specific introns to combine functionally essential parts of the involved genes while maintaining the reading frame of the two juxtaposed coding regions. Reciprocal translocations potentially give rise to two reciprocal gene fusions encoded by the two derivative chromosomes involved in the translocation event. In most cases however, only one of the fusion genes encodes a chimeric protein that is responsible for the oncogenic transformation. Chimeric proteins often deregulate cell growth or differentiation due to the combination of functional domains from both partners. In addition, one of the fusion partners may normally be silent in the hematopoietic lineage, or may only be expressed at a specific maturation stage, while the promoter driving the fusion gene is more ubiquitously active, resulting in deregulation of the fusion gene.

Most recurrent translocations are associated with specific leukemia subtypes suggesting that at least one of the affected genes normally plays a role in blood cell development. Functional dissection of the proto-oncogene products therefore may give insights into the regulation of proliferation and differentiation in the hematopoietic system. The *in vivo* oncogenicity of leukemia-specific fusion proteins is apparently restricted to hematopoietic cells, since expression of fusion proteins under control of ubiquitous promoters in mice generally leads to the development of hematopoietic malignancies only. Also, the malignant phenotype in these mice takes several weeks to months to occur, indicating that tumor formation requires additional mutations to complement the transgene.

4 The DEK-CAN and SET-CAN fusion genes in acute leukemia

The *CAN* gene, located on chromosome 9q34, is involved in at least two chromosomal rearrangements associated with acute leukemia. First, the recurrent t(6;9) is found with low frequency in acute myeloid leukemia (AML) and myelodysplastic syndrome (Rowley and Potter, 1976; Soekarman et al., 1992). The translocation fuses the *DEK* gene, on chromosome 6p23, to *CAN* and results in expression of a chimeric *DEK-CAN* mRNA from the 6p$^-$ derivative (Von Lindern et al., 1992a). DEK is a nuclear sequence-specific DNA-binding protein that recognizes the pets site, a DNA element in the human immunodeficiency virus type 2 (HIV-2) enhancer involved in upregulation of this promoter in response to T-cell or monocyte mitogenic stimulation. Divergent pets-like sites are also present in the promoters for myeloperoxidase and neutrophil elastase, potentially linking DEK to gene expression in immature neutrophils (Fu et al., 1997). Except for an N-terminal acidic region, DEK does not contain any known protein motifs (Figure 1). CAN is a nuclear pore complex (NPC) component, or nucleoporin, that contains multiple phenylalanine-glycine (FG) motifs characteristic for a group of NPC proteins (Fornerod et al., 1995). CAN is also called NUP214, for nucleoporin of 214 kDa (Kraemer et al., 1994).The central region of CAN contains several α-helical protein interaction domains. Most of CAN's FG repeats and one amphipathic helix are retained in the DEK-CAN fusion protein, which contains almost the entire DEK protein and the C-terminal two-thirds of CAN (Figure 1). DEK-CAN is located in the nucleus, resulting in the relocation of the C-terminal, FG repeat-rich region of CAN from the nuclear envelope to the nucleus (Fornerod et al., 1995).

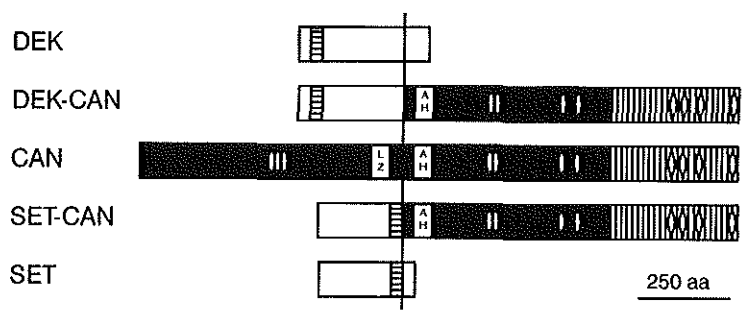

Figure 1. Structural features of CAN-derived fusion proteins

Fusion proteins created by t(6;9) and ?(9;9) contain the nucleoporin-specific FG repeat-rich C-terminus of CAN fused to DEK or SET. The fusion proteins and their normal counterparts are aligned on their fusion and break points (vertical line). Diamonds, FXF repeats; Vertical bars, FG repeats; LZ, coiled-coil and leucine zipper; AH, amphipathic helix; Horizontal bars, acidic regions.

A second chromosome rearrangement found in one patient with acute undifferentiated leukemia (AUL) fuses the same part of *CAN* to *SET*, located on chromosome 9q34 at the centromeric side of *CAN* and *c-ABL* (Von Lindern et al., 1992b). SET is a nuclear protein whose only common feature with DEK is an acidic region, that is also retained in the SET-CAN fusion protein (Figure 1). Like DEK-CAN, SET-CAN is a nuclear protein (Fornerod et al., 1995). SET, which has sequence similarity to nucleosome assembly protein 1 (NAP-1), was identified as a template activating factor (TAF-I) that is required for adenovirus DNA replication (Kellogg et al., 1995; Nagata et al., 1995). Therefore, SET may interact with core proteins complexed to the adenovirus genome and facilitate their dissociation from DNA, thereby rendering the DNA accessible for binding of replication proteins (Nagata et al., 1995). Furthermore, SET interacts specifically with B type cyclins (Kellogg et al., 1995) and inhibits the protein serine/threonine phosphatase PP2A (Li et al., 1996). PPA2 is involved in diverse cellular processes, such as cell cycle transition and DNA replication (Cohen, 1989; Mumby and Walter, 1993; Shenolikar and Nairn, 1991). Recently, SET was found to bind to MLL, a protein that is involved in many chromosome translocations associated with both myeloid and lymphoid acute leukemias (Adler and Tkachuk, 1996). MLL binds DNA in a non-sequence-specific manner and is thought to function as an architectural factor in transcription factor-DNA complexes (Tkachuk et al., 1992).

It is not clear by which mechanisms DEK-CAN and SET-CAN transform hematopoietic cells. Since DEK, SET and CAN are normally expressed in many cell types, including those of the hematopoietic system, ectopic expression of the fusion genes is probably not the main cause of transformation. More likely, the combination of functional domains of CAN with domains of SET or DEK leads to deregulation of the normal functions of one or both of the fusion partners. The studies described in Chapters 2 to 6 of this thesis aim at understanding the biological functions of CAN and the role of the CAN-derived fusion proteins in leukemogenic transformation.

For a better understanding of the mechanism by which DEK-CAN and SET-CAN contribute to leukemogenesis, it is useful to review other fusion proteins involved in human leukemias for which the affected genes have been cloned and characterized in some detail. Given what we and others found out about the structure and function of DEK, SET, and CAN, the fusion proteins could interfere with several basic cellular processes. The identification of DEK as a sequence-specific DNA-binding protein suggests that DEK-CAN could function as an altered transcription factor. Therefore, some prominent examples of fusion proteins that deregulate transcription will be discussed in section 5.1. SET also seems to be involved in transcription, or in replication, but the available data suggest that SET plays an indirect role by affecting chromatin structure. Thus, SET could belong to the super family of architectural factors, some of which have been implicated in tumorigenic fusion proteins (section 5.2). From the perspective of CAN, the involvement of an NPC component in an oncogenic fusion

Table 1. Gene fusions encoding chimeric oncoproteins

Translocation	Malignancy	Affected gene	Protein domain
Transcription factors			
t(1;19)(q23;p13.3)	pre-B acute lymphoblastic leukemia	*E2A* (19p13) *PBX-1* (1q23)	transactivation homeodomain
t(17;19)(q22;p13)	pro-B acute lymphoblastic leukemia	*E2A* (19p13) *HLF* (17q22)	transactivation bZIP
t(15;17)(q21;q11-22)	acute promyelocytic leukemia	*PML* (15q21) *RARα* (17q21)	CH-rich, coiled-coil DNA & RA binding
t(11;17)(q23;q21.1)	acute promyelocytic leukemia	*PLZF* (11q23) *RARα* (17q21)	POZ, zinc finger DNA & RA binding
t(5;17)(q32;q21)	acute promyelocytic leukemia	*NPM* (5q32) *RARα* (17q21)	oligomerization DNA & RA binding
t(8;21)(q22;q22)	acute myeloid leukemia	*AML1* (21q22) *ETO* (8q22)	runt homology zinc fingers
t(3;21)(q26;q22)	chronic myeloid leukemia, acute myeloid leukemia, myelodysplasia	*AML1* (21q22) *EVI1* (3q26)	runt homology zinc fingers
t(3;21)(q26;q22)	myelodysplasia	*AML1* (21q22) *EAP* (3q26)	runt homology unknown
t(3;21)(q26;q22)	acute myeloid leukemia	*AML1* (21q22) *MDS1* (3q26)	runt homology unknown
t(12;21)(p13;q22)	pre-B acute lymphoblastic leukemia	*TEL* (12p13) *AML1* (21q22)	helix-loop-helix runt homology
inv(16)(p13;q22)	acute myeloid leukemia	*CBFβ* (16q22) *MYH11* (16p13)	AML1-binding coiled-coil
Architectural factors			
t(12;15)(q15;q24)	lipoma	*HMGI-C* (12q15) novel gene (15q24)	AT hooks ST-rich
t(3;12)(q29;q15)	lipoma	*HMGI-C* (12q15) novel gene (3q29)	AT hooks LIM domains
t(4;11)(q21;q23)	acute lymphoblastic leukemia	*MLL* (11q23) *AF-4* (4q21)	AT hooks, MT SP-rich
t(9;11)(p22;q23)	acute myeloid leukemia	*MLL* (11q23) *AF-9* (9p22)	AT hooks, MT SP-rich
t(11;19)(q23;p13)	acute myeloid leukemia and acute lymphoblastic leukemia	*MLL* (11q23) *ENL* (19p13)	AT hooks, MT SP-rich

Table 1. Continued

Tyrosine kinases			
t(9;22)(q34;q11)	chronic myeloid leukemia and acute leukemia	*BCR* (22q11) *c-ABL* (9q34)	ST kin, multimers tyrosine kinase
t(5;12)(q33;p13)	chronic myelomonocytic leukemia	*TEL* (12p13) *PFGFRβ* (5q33)	helix-loop-helix tyrosine kinase
t(9;12)(q34;p13)	acute undifferentiated leukemia and acute myeloid leukemia	*TEL* (12p13) *ABL* (9q34)	helix-loop-helix tyrosine kinase
t(1;7)	gastric carcinomas	*TPR* (1) *MET* (7)	coiled-coil tyrosine kinase
?(1;1)	papillary thyroid carcinomas	*TPR* (1) *TRK* (1q)	coiled-coil tyrosine kinase
Nucleoporins			
t(6;9)(p23;q34)	acute myeloid leukemia and myelodysplasia	*DEK* (6p23) *CAN* (9q34)	unknown nup repeats
?(9;9)(q34;q34)	acute undifferentiated leukemia	*SET* (9q34) *CAN* (9q34)	unknown nup repeats
t(7;11)(p15;p15)	acute myeloid leukemia	*NUP98* (11p15) *HOXA9* (7p15)	nup repeats homeodomain

The protein domains indicated are those likely to be important in tumor pathogenesis. Abbreviations used: bZIP, basic region leucine zipper; CH, cysteine/histidine; RA, retinoic acid; POZ, protein-protein interaction domain; AT, adenine, thymine; ST, serine/threonine; LIM, cysteine-rich motif; MT, DNA-methyltransferase; SP, serine/proline; ST kin, serine/threonine protein kinase; nup, nucleoporin.

protein is intriguing. Thusfar, two other nucleoporins have been found as part of fusion proteins. First, TPR, which does not contain nucleoporin-specific repeat motifs, is fused to several partners with protein kinase activity. These and other examples of chimeric genes encoding activated tyrosine kinases will be reviewed in section 5.3. Second, the FG repeat-containing nuclear pore complex component NUP98 is fused to HOXA9 in AML. This fusion protein, and a brief overview of nucleocytoplasmic transport, will be described in section 5.4. Table 1 summarizes the discussed chromosome aberrations and the resulting fusion proteins.

5 Molecular themes in transformation by fusion proteins

5.1 Fused transcription factors

Transcription factors contain a DNA-binding motif that recognizes specific DNA sequences present in the regulatory regions of target genes. With their transactivation domains, transcription factors positively or negatively regulate RNA polymerase II activity by direct or indirect interaction with the basal transcription initiation complex. In order to allow either DNA binding or transcriptional activation, transcription factors often need to form homo- or heteromeric complexes. This process is mediated by protein interaction modules present in many transcription factors. Chromosome translocations often alter the structure of transcription factors, which leads to their activation. Why should transcription factors play such a central role in leukemogenesis? The neoplastic behavior of a malignant cell reflects altered patterns of transcription factor activity. One can speculate that mutations affecting cytoplasmic and cell surface membrane components of signal transduction pathways may be subject to a series of physiological checks and balances which tend to limit signal alterations of nuclear transcription factor activity. By contrast, mutations which directly affect transcription factor activity may be less easily regulated and hence more potently oncogenic (Green, 1992).

E2A fusions

In pediatric acute lymphoblastic leukemia (ALL) the translocation site is frequently located within the *E2A* gene on chromosome 19, which encodes the basic helix-loop-helix (HLH) DNA-binding immunoglobulin enhancer binding proteins E12 and E47 (Henthorn et al., 1990; Murre et al., 1989; Nelson et al., 1990). In two translocations involving *E2A*, t(17;19) and t(1;19), the N-terminal portion of E2A, which contains two transactivation motifs, is linked to a heterologous DNA-binding domain and a dimerization domain. In addition to the unique structure of these chimeric transcription factors, the *E2A* promoter directs expression of the fusion partners, that are not normally expressed in lymphoid cells.

In the t(1;19) translocation, found in a subset of childhood pre-B-cell acute leukemia, the 3' end of the chimeric gene contains the homeodomain and flanking sequences derived from *PBX1* (Kamps et al., 1991; Kamps et al., 1990; Nourse et al., 1990). Homeodomain proteins, such as PBX1, are sequence-specific transcription factors that regulate normal differentiation and development (McGinnis et al., 1984). By itself, PBX1 is incapable of transforming NIH 3T3 cells and it requires fusion with E2A to activate its oncogenic potential (Kamps et al., 1991). Similarly, in reporter systems using the PBX1 consensus sequence in lymphoid cells, the fusion protein strongly activates transcription whereas PBX1 does not (Van Dijk et al., 1993). Surprisingly, the

PBX1 homeodomain was found to be dispensable for transformation (Monica et al., 1994). A stretch of conserved amino acids located C-terminal of the PBX1 homeodomain is involved in cooperative DNA binding with a subset of HOX proteins (Chang et al., 1995) and is required for transformation (Monica et al., 1994). This region could contribute to oncogenesis by heterodimerization with HOX proteins which direct E2A-PBX1 to regulatory sequences of appropriate target genes. Alternatively, this region of E2A-PBX1 could mediate the formation of non-DNA-binding complexes that sequester tumor-suppressing factors.

Introduction of the E2A-PBX1 gene in mice induces acute hematopoietic malignancies, albeit of cell types that differ from those affected in the human disease. Transgenic mice expressing E2A-PBX1 under control of the immunoglobulin heavy chain enhancer demonstrate profound disturbances in T and B cell development, including increased proliferation and apoptosis, and the animals die before five months of age with T-cell lymphomas (Dedera et al., 1993). On the other hand, mice reconstituted with bone marrow cells infected with a retrovirus carrying a cDNA encoding E2A-PBX1 develop AML after three to eight months (Kamps and Baltimore, 1993). Murine myeloid progenitor cells that are infected *in vitro* with the E2A-PBX1 retrovirusses are strongly retarded in their differentiation without loosing their growth-factor dependence (Kamps and Wright, 1994). These data suggest that a principal role for E2A-PBX1 in leukemia is to block hematopoietic differentiation.

In pro-B-cell ALL involving the t(17;19) translocation, the transactivation domain of E2A is linked to the basic leucine zipper (bZIP) DNA-binding and dimerization domains of hepatic leukemia factor (HLF) (Hunger et al., 1992; Inaba et al., 1992). HLF belongs to the proline- and acidic amino acid-rich (PAR) subfamily of bZIP proteins (Drolet et al., 1991), binds DNA, and activates transcription in a tissue-specific manner. Blocking E2A-HLF activity in a t(17;19)-positive human leukemia cell line results in rapid apoptosis, suggesting that E2A-HLF affects cell survival rather than cell growth. Moreover, when introduced into murine pro-B lymphocytes, E2A-HLF reverses both interleukin-3-dependent and p53-mediated apoptosis (Inaba et al., 1996). E2A-HLF forms homodimers that bind to the HLF consensus site and transactivate reporter gene expression in a wide variety of cell types (Hunger et al., 1992; Inaba et al., 1994), suggesting that E2A-HLF homodimers could directly regulate the expression of genes required for cell survival. Since mutants lacking HLF-mediated DNA-binding can also promote cell survival, E2A-HLF may have an additional capacity to block apoptosis by heterodimerizing with other bZIP transcription factors that normally bind to the HLF consensus sequence and regulate programmed cell death in pro-B-cells (Hunger et al., 1994; Inukai et al., 1996).

RARα fusions

A t(15;17) occurs in virtually all cases of a subtype of AML known as acute promyelocytic leukemia (APL) (Rowley et al., 1977). The translocation fuses the gene for the retinoic acid-α receptor (*RARα*) on chromosome 17 to the promyelocytic leukemia gene (*PML*) on chromosome 15 (De Thé et al., 1991; Kakizuka et al., 1991). Retinoic acid (RA) has significant effects on development, differentiation and proliferation, and RARα is a nuclear, inducible transcription factor mediating these effects (reviewed in Gillard and Solomon, 1993). The expression of a functionally altered RA receptor explains the response of t(15;17) promyelocytic blasts to all-*trans* RA as a differentiating agent *in vitro* and *in vivo* (Chomienne et al., 1990; Huang et al., 1988). PML was recently discovered to suppress growth and transformation (Wang et al., 1996d) and belongs to a family of nuclear proteins which share N-terminal cysteine/histidine-rich clusters, including the so-called RING domain (Lovering et al., 1993; Reddy et al., 1992). Immediately C-terminal to the cysteine cluster, PML contains a coiled-coil dimerization motif, and its N-terminus contains a proline-rich putative transactivation domain (De Thé et al., 1991; Kakizuka et al., 1991; Kastner et al., 1992). The PML-RARα hybrid protein contains most of the native PML protein fused to the DNA- and hormone-binding domains of RARα. The PML-RARα fusion protein also retains RARα's interface of dimerization with retinoid X receptors (RXRs) (reviewed in Kastner et al., 1994), a family of retinoid receptors which act as co-factors for other nuclear receptors.

The coiled-coil region of PML directs the formation of PML-RARα homodimers and PML-RARα/PML heterodimers *in vitro* (Kastner et al., 1992; Perez et al., 1993). PML-RARα homodimers can bind to the RA-responsive elements on the DNA, in contrast to RARα itself that requires the RXR dimerization partner (Jansen et al., 1995; Perez et al., 1993). Furthermore, PML-RARα demonstrates promoter and cell-type specific alterations in RARα-mediated transcriptional activation compared with wild-type RARα (De Thé et al., 1991; Kakizuka et al., 1991; Kastner et al., 1992; Pandolfi et al., 1991). Importantly, PML-RARα inhibits transactivation of some RA target proteins by RARα-RXRα heterodimers in a dominant fashion (Jansen et al., 1995). These properties of PML-RARα may contribute to the transformed phenotype of APL cells (Weis et al., 1994). In addition, PML-RARα could act in a dominant-negative way to disruption the growth suppressing activity of PML. PML is normally localized in specific nuclear subdomains, so-called nuclear bodies. In APL cells, these nuclear bodies are disrupted and PML-RARα and PML co-localize in poorly characterized microspeckled nuclear structures (Dyck et al., 1994; Kastner et al., 1992; Koken et al., 1994; Weis et al., 1994).

PML-RARα blocks vitamin D_3-induced differentiation of myeloid precursor cell lines and reduces the frequency of commitment to apoptosis upon growth factor deprivation (Grignani et al., 1993; Rogaia et al., 1995). The PML coiled-coil domain is

indispensable for the blocking activity of PML-RARα on differentiation, whereas the RARα DNA-binding domain only contributes partially (Grignani et al., 1996). Recently, PML-RARα transgenic mice in which the transgene is specifically expressed in promyelocytes were found to have altered myeloid development and an increased frequency of AML after a long latency period (Grisolano et al., 1997). In conclusion, the capacity of PML-RARα to regulate retinoid acid target genes, to bind RXR and PML *in vitro* and to disrupt PML nuclear bodies *in vivo* has led to the hypothesis that PML-RARα might interfere with the function of a variety of nuclear proteins that include RARα, PML, other RXR-dependent nuclear receptors and other components of the nuclear bodies.

In a variant translocation t(11;17), found in a small subset of APL patients, RARα is fused to the N-terminal part of promyelocytic leukemia zinc finger (PLZF) protein (Chen et al., 1993). PLZF belongs to a family of proteins with a highly conserved N-terminal POZ domain, which mediates protein-protein interactions and nuclear localization, and contains a C-terminal Kruppel-like zinc finger DNA-binding domain. Like PML, PLZF is a growth suppressor. (Chen et al., 1993). Moreover, PLZF and PML partly co-localize to the same nuclear bodies and interact with each other, suggesting a functional relationship between these two proteins (Koken et al., 1996). The POZ domain mediates the formation of PLZF-RARα homodimers and PLZF-RARα/PLZF heterodimers, whereas heterodimerization of PLZF-RARα with RXRα is mediated by the RARα sequence. It was shown that the POZ domain is primarily responsible for the inhibitory activity of PLZF-RARα on transactivation by the wild-type RARα/RXRα (Chen et al., 1994; Dong et al., 1996; Licht et al., 1996). Therefore, PLZF-RARα may play a role in leukemogenesis by antagonizing actions of both RA receptors and PLZF, and possibly other POZ-domain-containing regulators (Dong et al., 1996). Other parallels with PLM-RARα are the inhibition of terminal differentiation of hematopoietic precursor cell lines by PLZF-RARα and localization to the characteristic microspeckled structures (Ruthardt et al., 1996).

In a third translocation found in APL with t(5;17), RARα is fused to nucleoplasmin (NPM), a protein involved in ribosome assembly that forms oligomers *in vivo* (Chan and Chan, 1995; Redner et al., 1996b). Structural as well as functional similarities between NPM-RARα and the other two RARα fusion proteins suggests that the mechanisms by which they cause APL are similar. This conclusion is illustrated by the finding that NPM-RARα blocks differentiation of myeloid precursor U937 cells (Redner et al., 1996a). The occurrence of identical RARα sequences in three different fusion proteins demonstrates the importance of an altered RARα receptor in the pathogenesis of APL. Remarkably, the sole identified common feature of at least three different fusion partners of RARα in APL - PML, PLZF, and NPM - is their ability to mediate oligomerization.

CBF fusions

A frequent target of chromosome translocations in leukemia is the core-binding factor (CBF) transcription complex, which consists of the acute myeloid leukemia 1 (AML1) protein and core-binding factor β (CBFβ). AML1 contains an N-terminal DNA-binding domain, which is homologous to the *Drosophila* segmentation gene product runt, and a C-terminal transactivation domain (Daga et al., 1992; Erickson et al., 1992; Kania et al., 1990). From the *AML1* gene two representative forms of proteins - AML1a and AML1b - are generated by alternative splicing, that both have the runt homology domain (RHD) but, unlike the full-length AML1b, AML1a lacks the transcriptional activation domain (Miyoshi et al., 1995). AML1a antagonizes both transactivation and myeloid cell differentiation by AML1b, and may function as a regulator of the activity of full-length AML1b (Tanaka et al., 1995b).

The DNA-binding site for AML1, called core-enhancer motif, is present in the regulatory sequences of a variety of hematopoietic-specific genes, such as those for T-cell receptor, myeloperoxidase, neutrophil elastase, interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor-1 (GM-CSF), and colony-stimulating factor-1 (CSF-1) receptor (Hiebert et al., 1996). Transient transfection studies have shown that AML1 can activate promoter-enhancer constructs from some of these genes. The crucial role of AML1 in hematopoietic differentiation is confirmed by gene-disruption experiments, since mice lacking AML1 die during embryogenesis because of a defect in the establishment of definitive hematopoiesis of all lineages (Okuda et al., 1996a; Wang et al., 1996b).

At least three different chromosome translocations - t(8;21), t(3;21), and t(12;21) -

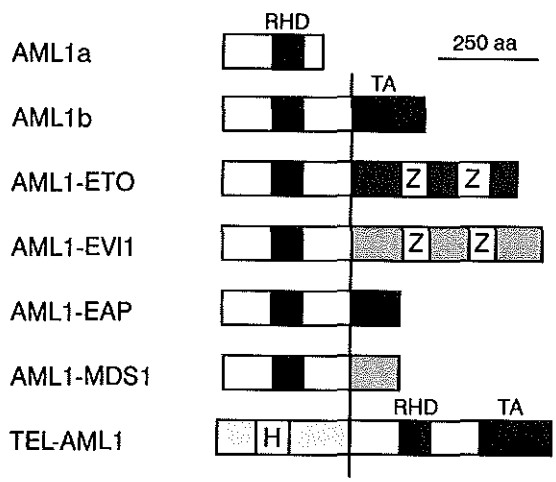

Figure 2. Fusion proteins involving transcription factor AML1

Structural features of two splice forms of AML1, AML1a (truncated isoform) and AML1b (full-length AML1 protein). Fusion proteins AML1-ETO, AML1-EVI1, AML1-EAP, and AML1-MDS1 have lost the AML1 transactivation domain. In TEL-AML1, the TEL gene is fused to the N-terminal end of the entire AML1 gene. RHD, runt homology domain; TA, transactivation; Z, zinc fingers; H, helix-loop-helix.

share a breakpoint in AML1, at 21q22. AML1 was first identified through its involvement in the AML-associated t(8;21), which juxtaposes 5' sequences of *AML1* to 3' sequences of *ETO* (for eight twenty-one) on the derivative 8 chromosome. The chimeric gene product contains the RHD of AML1 fused to near full-length ETO (Figure 2), a putative zinc finger-containing transcription factor that can transform fibroblasts (Erickson et al., 1992; Miyoshi et al., 1993; Wang et al., 1996a). AML1-ETO retains the ability to interact with the enhancer core DNA sequence, but suppresses AML1-dependent transactivation (Meyers et al., 1993; Meyers et al., 1995). Thus, transformation by AML1-ETO may result from dominant-negative activity on wild-type AML1. Further evidence for this hypothesis is provided by *in vivo* expression of AML1-ETO by homologous recombination in mice. Embryos heterozygous for the AML1-ETO fusion gene die in midgestation with a phenotype very similar to that caused by homozygous disruption of AML1. Culture of hematopoietic precursor cells from these embryos revealed an increase in dysplastic colonies of macrophage or mixed origin, indicating an additional gain-of-function activity of AML1-ETO as activator of some myeloid cells (Okuda et al., 1996b; Yergeau et al., 1996). An alternative strategy, which will allow expression of the fusion gene only in adult hematopoietic cells, should help to better address the role of the fusion protein in leukemogenesis.

The consequence of three different t(3;21)(q26;q22) translocations is similar to that of t(8;21), replacement of the AML1 transactivation domain with sequences from another protein (Figure 2). The RHD-encoding part of *AML1* is fused to either the *EVI1* gene, encoding a zinc finger-containing transcription factor, or to *EAP*, which codes for the abundant ribosomal protein L22, or to *MDS1*, a gene of unknown function (Mitani et al., 1994; Nucifora et al., 1993; Nucifora et al., 1994). Like AML1-ETO, AML1-EVI1, AML1-EAP, and AML1-MDS1 fusion proteins all dominantly repress transactivation by intact AML1 (Meyers et al., 1995; Zent et al., 1996). Furthermore, AML1-EVI1, as well as AML1a and EVI1, block granulocytic differentiation of 32DCL3 myeloid precursor cells without abrogating IL-3 requirement for growth (Morishita et al., 1992a, 1992b; Tanaka et al., 1995a; Tanaka et al., 1995b).

Rearranged forms of the *AML1* gene are not only associated with myeloid leukemias, but also with lymphoid leukemias. In t(12;21), associated with childhood pre-B cell ALL, almost the entire AML1 protein is fused to the N-terminal part of TEL (Translocation, Ets, Leukemia), a member of the Ets family of transcription factors (Golub et al., 1995; Romana et al., 1995). The N-terminus of TEL contains a conserved domain, which has weak homology to the helix-loop-helix (HLH) domains of transcription factors such as myc and myoD, that mediates oligomerization of TEL and TEL-derived fusion proteins (Jousset et al., 1997). The TEL-AML1 fusion is unique in that the AML1 RHD and transactivation domains are both preserved in the chimeric protein (Figure 2), and that the fusion transcript is expressed from the *TEL* promoter. Despite the presence of the AML1 transactivation domain, TEL-AML1 efficiently

interferes with AML1-dependent transactivation and TEL's HLH domain is essential for this repression (Hiebert et al., 1996). These data suggest that the inhibition of expression of AML1 target genes is critical for B-cell leukemogenesis. Another intriguing finding is the deletion of the *TEL* allele from the cytogenetically normal copy of chromosome 12 in part of the patients with a *TEL* translocation (Golub et al., 1995; Kobayashi et al., 1994). Recent findings suggest that TEL may antagonize the function of some transcriptional activators of the Ets family (Ringold et al., 1996). Thus, TEL-AML1 may also contribute to hematopoietic transformation through interference with wild-type TEL function.

The binding partner of AML1 in the CBF transcription complex, CBFβ, was found to be targeted by inversion (16)(p13; q22) associated with a subtype of AML (Liu et al., 1993). CBFβ does not contact DNA directly, but increases the DNA-binding affinity of AML1 by heterodimerization through the RHD and is required for transactivation by AML1 (Meyers et al., 1993; Wang et al., 1993). Inversion 16 results in the fusion of *CBFβ* to a smooth muscle myosin heavy chain gene, *MYH11*. The chimeric product, which still interacts with AML1, contains most of the CBFβ protein and the coiled-coil dimerization motif of MYH11 (Liu et al., 1993). Depending on the promoter, CBFβ-MYH11 can either enhance or suppress AML1 activity and it has been proposed that the large size of the CBFβ-MYH11/AML1 transcription factor complex may interfere with adjacent co-activator or repressor proteins required for normal CBF function (Castilla et al., 1996; Liu et al., 1994). Mouse embryos heterozygous for CBFβ-MYH11 lacked definitive hematopoiesis (Castilla et al., 1996). This phenotype is strikingly similar to that resulting from homozygous deletions of either AML1 (Okuda et al., 1996a; Wang et al., 1996b) or CBFβ (Wang et al., 1996c), and from *in vivo* AML1-ETO expression (Okuda et al., 1996b; Yergeau et al., 1996). This observation is consistent with a dominant negative function of the CBFβ-MYH11 fusion oncogene on CBF. An impairment of primitive hematopoiesis was also observed, however, suggesting a possible additional function of CBFβ-MYH11 (Castilla et al., 1996).

In conclusion, the frequent targeting of the AML1/CBFβ complex by leukemia-associated chromosomal rearrangements suggests that alteration of its activity leads to the disruption of AML1-mediated signals, which are critical for normal growth or differentiation of hematopoietic cells.

5.2 Architectural factors in fusion proteins

Transcriptional regulation in eukaryotes is based on the synergistic action of several DNA-binding transcription factors, assembled on a given enhancer or promoter sequence. An additional mechanism for achieving a high level of specificity and gene activation is the assembly of a stereospecific nucleoprotein complex. This process

requires proteins that function as architectural components in the nuclear scaffold through protein-protein and protein-DNA interactions (for review see O'Neill et al., 1994). These proteins differ from classical transcription factors in that they lack activation domains and have little if any sequence specificity; instead they bind to the minor groove of DNA causing sharp bends in the double helix (Grosschedl et al., 1994; Paull et al., 1993; Pil et al., 1993).

The architectural proteins belonging to the high-mobility group (HMG) family of proteins are abundant, heterogeneous, nonhistone components of chromatin (Bustin et al., 1990; Grosschedl et al., 1994). HMG proteins are divided into three distinct classes, the HMG domain containing HMG1/HMG2, the active chromatin-associated HMG14/HMG17, and the HMGI proteins, that bind DNA sequences rich in adenine and thymine with domains termed AT hooks (Reeves and Nissen, 1990). Additional proteins carrying AT hook motifs were discovered and they form a new class of proteins that are involved in transcriptional regulation of genes that contain or are near AT-rich DNA. They are proposed to bind in the minor groove within core elements of enhancer sites, resulting in tortional changes of the DNA to facilitate binding of transcription factors within the major groove (Tkachuk et al., 1992). HMG1/HMG2 as well as HMGI proteins were shown to physically and functionally interact with sequence-specific transcription factors by enhancing DNA binding and transcriptional activation (Du et al., 1993; Oñate et al., 1994; Thanos and Maniatis, 1992; Zappavigna et al., 1996; Zwilling et al., 1995). A number of studies have revealed an association between increased expression of HMGI proteins and transformation (Berlingieri et al., 1995; Giancotti et al., 1989; Ram et al., 1993). Interestingly, two genes encoding architectural factors that contain AT hook motifs, *HMGI-C* and *MLL*, are translocated with multiple chromosomal partners in mesenchymal neoplasms and leukemias, respectively.

HMGI-C fusions

Rearrangements of chromosome region 12q14-15 are consistently associated with a number of benign tumors, mainly of mesenchymal origin (Schoenberg Fejzo et al., 1995). The affected gene was identified as *HMGI-C*, coding for an HMGI protein (Patel et al., 1994). A variety of chromosome translocations results in fusion of the AT hooks of HMGI-C to novel C-terminal sequences. In a lipoma containing a t(12;15)(q15;q24) a highly acidic, serine- and threonine-rich domain that could contribute a transcriptional activator was fused to HMGI-C (Ashar et al., 1995). In a second lipoma carrying t(3;12)(q29;q15), two tandemly arrayed LIM domains, involved in protein-protein interactions, may recruit other transcriptional activators to the DNA sites bound by the AT hooks of HMGI-C (Ashar et al., 1995; Schoenmakers et al., 1995). These studies suggest that a number of transcriptional regulatory domains can be placed downstream of the HMGI-C AT hooks and contribute to the pathobiology of lipomas. Truncation of HMGI-C may also contribute to tumorigenesis, since in a leiomyoma with

inv(12)(q14-15;q24) only ten codons of the mitochondrial aldehyde dehydrogenase gene were linked to *HMGI-C* (Kazmierczak et al., 1995). The association of chromosomal rearrangements in bands 12q14-15 with benign solid tumors of mesenchymal origin implicates *HMGI-C* as a key regulator in adipogenesis and mesenchyme differentiation.

MLL fusions

The *MLL* gene (also called *ALL-1*, *HRX* or *HTRX*), located on chromosome 11q23, is frequently affected by a variety of chromosomal rearrangements that occur in ALL and in *de novo* and secondary AML (Djabali et al., 1992; Gu et al., 1992; Tkachuk et al., 1992; Ziemin-van der Poel et al., 1991). The *MLL* gene encodes a very large protein (Figure 3) that shares three regions of similarity with the trithorax protein of *Drosophila melanogaster*: two central zinc finger motifs and a 210-amino acid C-terminal segment (Gu et al., 1992; Tkachuk et al., 1992). The N-terminal region of MLL contains three AT hook motifs, a region with homology to the non-catalytic domain of DNA-methyltransferase (MT), and a region similar to the 70 kDa protein of U1 small nuclear ribonucleoprotein particles (Domer et al., 1993; Ma et al., 1993; Tkachuk et al., 1992). Studies of MLL knock-out embryos showed that MLL is required for normal hematopoiesis and for the maintenance of expression of Hox genes, which have been implicated in development and hematopoietic differentiation (Hess et al., 1996). MLL's involvement in acute leukemias of multiple lineages suggests that it is an important gene in early lymphoid-myeloid differentiation.

More than 20 different reciprocal translocations involving the MLL gene have been reported, most common of which are t(4;11), t(9;11), and t(11;19) (Thirman et al.,

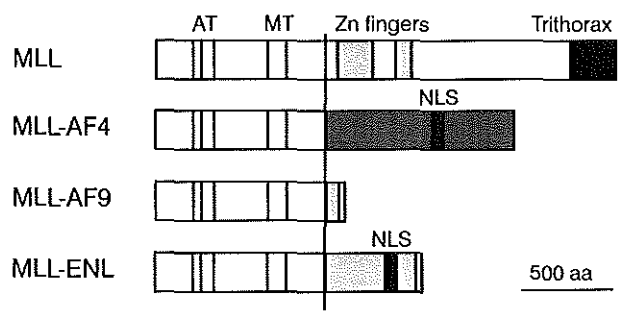

Figure 3. Chimeric proteins containing the architectural factor MLL

Schematic representation of three different fusion proteins composed of the N-terminal region of MLL and the C-terminal regions of one of three different partner proteins, AF-4, AF-9 or ENL, resulting from t(4;11), t(9;11) and t(11;19), respectively. The C-terminal regions of AF9 and ENL are homologous. The vertical line indicates the fusion point in each chimeric protein. AT, AT hooks; MT, DNA-methyltransferase; NLS, nuclear localization sequence.

1993). The fusion genes encode chimeric proteins in which a similar N-terminal part of MLL, including the AT hooks and MT domain, but lacking the zinc-finger motifs and C-terminal trithorax homology, is fused to novel protein sequences (Downing et al., 1994; Rowley, 1992). The role of the MLL portion in the fusion proteins is not clear. On the one hand, AT hooks bind the minor groove of DNA and may allow other transcription factors to gain access (Tkachuk et al., 1992). On the other hand, the MT domain may allow the MLL fusion proteins to modify the methylation status of DNA and thereby affect transcription.

Several of the genes that participate in 11q23 translocations have been cloned and sequenced (reviewed in Rubnitz et al., 1996). *AF4*, localized on chromosome 4q21, encodes a serine/proline-rich protein that contains a nuclear localization sequence (NLS) (Gu et al., 1992; Morrissey et al., 1993). *AF-9* (band 9p22) and *ENL* (band 19p13) encode homologous proteins that also contain NLSs and are rich in serine and proline, suggesting that these three proteins may function in the regulation of transcription (Nakamura et al., 1993; Rubnitz et al., 1994). Not all chimeric products are expected to act through the same mechanism, since sequence analysis of additional *MLL* partner genes suggests that there are no common structural features shared by all of them. Recently, in several cases of AML that lacked 11q23 translocations, partial internal duplications of *MLL* were found resulting in dissociation of the AT hooks and MT domain from the remainder of the protein (reviewed in Schichman et al., 1995). Uncoupling of N-terminal domains of MLL from regulatory C-terminal regions may be a crucial alteration in the oncogenic transformation of MLL.

The subtype of the leukemia correlates with specific MLL fusion proteins. For example, t(4;11), which generates the MLL-AF4 fusion protein, is found predominantly in ALL (Djabali et al., 1992; Domer et al., 1993; Gu et al., 1992; Morrissey et al., 1993; Nakamura et al., 1993), whereas t(9;11) is mainly associated with AML and fuses AF9 to MLL (Iida et al., 1993; Nakamura et al., 1993). However, tumors of different phenotype can have similar or identical MLL chimeric proteins, indicating that the tumor phenotype is not dictated by the type of MLL fusion product alone (Corral et al., 1993; Lo Coco et al., 1993; Thirman et al., 1993). The timing of the MLL-associated chromosomal translocation compared to additional mutations, and the precise stage of commitment of the cell in which the translocation occurs may combine to determine lineage of the tumor (Corral et al., 1996).

To mimic t(9;11) in mice, an *MLL-AF9* fusion gene was made by homologous recombination in embryonic stem cells, so that expression of the fusion gene occurred from endogenous *MLL* promoter elements (Corral et al., 1996). Chimeric mice carrying the fusion gene develop AML closely resembling the human disease, with a latency period of four months. A truncation of MLL, generated in a similar way, did not lead to disease, indicating that at least in mice the addition of functional domains from a fusion partner is essential for leukemogenesis.

5.3 Fusion proteins with tyrosine kinase activity

Tyrosine kinases are important in the transduction of signals regulating cell proliferation and differentiation. Abnormal expression of tyrosine kinases often leads to malignant transformation. Mutant protein tyrosine kinases include transmembrane growth factor receptors (e.g., the EGF receptor), membrane-associated signal transduction molecules (e.g., src), and soluble tyrosine kinases (e.g., fps). Truncation of protein tyrosine kinases often leads to mutant versions that deliver a continuous rather than a ligand-regulated mitogenic signal. Another way to activate some proteins containing tyrosine kinase activity is by fusion to a protein with an oligomerization domain.

BCR-ABL

The chimeric BCR-ABL oncogene, formed by the t(9;22) Philadelphia chromosome translocation, is present in almost all cases of chronic myelogenous leukemia (CML), 25-50% of cases of adult B-lineage ALL and about 5% of cases of AML (Kurzrock et al., 1988). Two different fusion proteins can be produced which differ in their BCR sequences. The $p210^{BCR\text{-}ABL}$ protein is associated with CML, and also present in about one-third of Philadelphia-positive ALL cases (Groffen et al., 1984; Konopka et al., 1984; Shtivelman et al., 1985). In the other cases a smaller $p190^{BCR\text{-}ABL}$ is found, which has a higher intrinsic tyrosine kinase activity and transforming potential (Chan et al., 1987; Clark et al., 1988; Hermans et al., 1987; Kurzrock et al., 1987).

c-ABL, the cellular homolog of the viral oncogene *v-ABL*, encodes a nuclear tyrosine kinase that negatively regulates growth (Sawyers et al., 1994; Van Etten et al., 1989). Fusion to BCR (Figure 4) relocates almost the entire ABL protein from the nucleus to the cytoplasm (Van Etten et al., 1989; Wetzler et al., 1993), exposing a now constitutively active tyrosine kinase to a new range of substrates. Since the growth suppression by ABL requires nuclear localization, the changed cellular location of the activated ABL kinases may contribute to leukemogenesis (Sawyers et al., 1994). BCR-ABL binds and phosphorylates proteins of the RAS signalling pathway and may also affect proteins involved in other growth-factor signalling pathways (summarized in Butturini et al., 1996).

In normal cells, BCR is widely expressed and BCR-deficient mice have defects in polymorphonuclear neutrophil function (Voncken et al., 1995b). The N-terminus of BCR contains a serine/threonine kinase domain (Maru and Witte, 1991) and a domain that binds to the SRC homology region 2 (SH2) domain of ABL (Muller et al., 1992). Its C-terminal third, that is not present in the BCR-ABL fusions, contains a GTPase-activating protein (GAP) activity for $p21^{rac}$ (Diekmann et al., 1991). Furthermore, the N-terminal 60 amino acids of BCR, which have an α-helical coiled-coil structure, mediate tetramerization of BCR-ABL proteins and are important for activation of the ABL kinase activity and transforming potential of BCR-ABL (McWhirter et al., 1993; Muller et al.,

1991). The oligomerization domain of BCR also mediates heterodimerization between BCR and BCR-ABL, so it is possible that interference of BCR-ABL with the normal function of BCR contributes to transformation (Campbell et al., 1990).

Much is known from experimental models about the growth-stimulatory properties of BCR-ABL that correlate with its ability to expand the myeloid compartment in CML. Firstly, introduction of the *BCR-ABL* gene into hematopoietic cell lines of many different lineages (myeloid, B or T lymphoid) renders them growth factor independent and tumorigenic in animals (Cook et al., 1985; Daley and Baltimore, 1988; Hariharan et al., 1988; Mandanas et al., 1992; Pendergast et al., 1993; Sirard et al., 1994). Secondly, expression of $p210^{BCR\text{-}ABL}$ was found to inhibit apoptosis and inappropriately prolong cell survival of human CML cells and transfected murine cell lines (Bedi et al., 1994; McGahon et al., 1994). Thirdly, mice expressing BCR-ABL proteins, either by retroviral infection of bone marrow cells or introduction of the transgene into the germline, develop diverse leukemias resembling the human disease (Daley et al., 1990; Elefanty et al., 1990; Groffen et al., 1993; Heisterkamp et al., 1990; Kelliher et al., 1991; Kelliher et al., 1990; Voncken et al., 1995a).

N-terminal TEL fusions

Two translocations resulting in the fusion of the N-terminal HLH domain of TEL to protein tyrosine kinases have been described. Firstly, in t(5;12), associated with chronic myelomonocytic leukemia, TEL is fused to the transmembrane and tyrosine kinase domains of the platelet-derived growth factor receptor β (PDGFRβ) (Golub et al., 1994). TEL-PDGFRβ renders a murine hematopoietic cell line IL-3 independent, presumably by activating PDGFRβ-dependent signaling pathways. Both the TEL HLH domain and the PDGFRβ tyrosine kinase activity are required for the transformation (Carroll et al., 1996). HLH-induced self-association was shown to be essential for the constitutive activation of the protein kinase activity and mitogenic properties of TEL-PDGFRβ

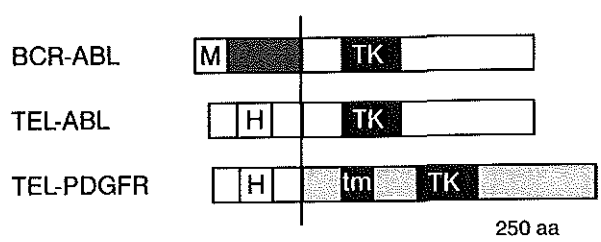

Figure 4. Structure of fusion proteins involving tyrosine kinases

Fusion proteins involving the ABL and PDGFRβ tyrosine kinases are aligned on their breakpoints. BCR-ABL is created by t(9;22), TEL-ABL by t(9;12), and TEL-PDFGRβ by t(5;12). TK, tyrosine kinase domain; H, helix-loop-helix dimerization motif; TM, transmembrane domain.

(Jousset et al., 1997). Apparently, oligomerization by TEL mimics ligand-dependent dimerization of the normal receptor, which is sufficient for tyrosine kinase activation and association with effector molecules.

Secondly, t(9;12)(q34;p13) associated with AUL and AML, links TEL to ABL (Golub et al., 1996; Papadopoulos et al., 1995). Both the tyrosine kinase and transforming activities of TEL-ABL are impaired by deletion of the TEL oligomerization domain (Golub et al., 1996). Because of the similarities to BCR-ABL, TEL-PDGFRβ and TEL-ABL are postulated to function by activation of similar signal transduction pathways (Sawyers, 1997).

TPR fusions

The translocated promoter region (*TPR*) gene appears in oncogenic fusions with the tyrosine kinase domains of the *MET* and *TRK* proto-oncogenes in gastric carcinomas and thyroid cancers, respectively (Greco et al., 1992; Soman et al., 1991). TPR is an NPC protein without FG repeats. It contains an exceptionally long predicted coiled-coil domain and is thought to be a structural component of the cytoplasmic fibrils of the NPC (Byrd et al., 1994). Fusion of the N-terminal coiled-coil of TPR to MET results in a constitutively active tyrosine kinase that causes malignancies in transgenic mice (Liang et al., 1996). A leucine zipper motif within TPR mediates homodimerization of the TPR-MET protein and is essential for the transforming activity of the fusion protein (Rodrigues and Park, 1993). It is possible that the TPR moiety of the fusion protein contributes more than an oligomerization domain. If TPR fusions are still able to bind to the NPC, they could interfere with nucleocytoplasmic transport by phosphorylating NPC components or transport substrates (Byrd et al., 1994).

5.4 Repeat-containing nucleoporins in fusion proteins

The NPC is a supramolecular structure that spans the nuclear envelope and mediates bidirectional transport of proteins and ribonucleoproteins (RNPs) between the cytoplasm and the nucleus (recently reviewed by Görlich and Mattaj, 1996). Multiple copies of an estimated 100 different proteins, named nucleoporins, are present at distinct positions within the NPC. A number of these proteins contain degenerated amino acid repeat motifs, that have an FG dipeptide core (Fabre and Hurt, 1994; Panté and Aebi, 1994; Rout and Wente, 1994). Studies in yeast mutants have established a role for several nucleoporins in mRNA export or nuclear protein import, or both, as well as in NPC morphology and distribution (reviewed by Doye and Hurt, 1995). Three vertebrate nucleoporins have been implicated in malignancies by gain-of-function mutations. Fusion proteins involving TPR, an NPC component that does not contain nucleoporin-specific FG repeat sequences, play a role in the oncogenic activation of

tyrosine kinases and are discussed above. To date, two repeat-containing nucleoporins have been implicated in leukemia by gene fusion, NUP98 and CAN.

NUP98-HOXA9

The translocation t(7;11)(p15;p15) is very rare, but has primarily been observed in AML. It creates a fusion between the *HOXA9* gene on chromosome 7 and the nucleoporin gene *NUP98* on chromosome 11. *NUP98* encodes an FG repeat-containing nucleoporin that is located on the nucleoplasmic side of the NPC (Radu et al., 1995b). The predicted fusion protein contains the repeat-rich N-terminal half of NUP98 and most of the coding region of the homeobox gene HOXA9, including its homeodomain (Borrow et al., 1996; Nakamura et al., 1996a). The murine *Hoxa9* gene was also found to be activated by proviral integration in myeloid leukemias in BXH-2 mice (Nakamura et al., 1996b), identifying this gene as an important myeloid leukemia gene in both man and mouse.

The presence of the FG repeats of both NUP98 and CAN in the NUP98-HOXA9, and DEK-CAN and SET-CAN fusion proteins, respectively, argues for an important contribution of these sequences to the development of leukemia. The peptide repeat domains function as NPC binding sites for cytosol-mediated docking of import substrates, at least *in vitro* (Iovine et al., 1995; Kraemer et al., 1995; Radu et al., 1995a; Radu et al., 1995b; Rexach and Blobel, 1995). A heterodimeric nuclear import receptor, consisting of importin β (also called p97 or karyopherin β) and importin α (also called karyopherin α or NLS receptor) binds to an NLS-bearing protein in the cytoplasm via the NLS-binding domain of importin α (Adam and Adam, 1994; Adam and Gerace, 1991; Görlich et al., 1994; Moroianu et al., 1995a; Weis et al., 1995). Importin β is predominantly localized at the NPC where it acts as an adaptor between importin α and repeat-containing nucleoporins (Chi et al., 1995; Görlich et al., 1995; Moroianu et al., 1995b). Translocation of the ligand-bound importin complex through the pore is driven by the GTP cycle of the small nuclear GTPase Ran (Melchior et al., 1993; Moore and Blobel, 1993), and may occur by repeated docking and undocking of importin β to an array of FG repeat-containing nucleoporins, including NUP98 and CAN, that line the NPC (Nehrbass and Blobel, 1996; Rexach and Blobel, 1995). At the nuclear side of the NPC, dissociation of the importin heterodimer is mediated by direct binding of RanGTP to importin β (Görlich et al., 1996b; Rexach and Blobel, 1995). The NLS protein and importin α are released into the nuclear interior, whereas importin β remains bound to the NPC (Görlich et al., 1995; Moroianu et al., 1995b). The gradient in Ran's nucleotide binding state is generated by the nuclear GDP/GTP exchange factor for Ran, RCC1, and cytoplasmic RanGAP, the Ran-specific GTPase activating protein (Bischoff et al., 1994; Bischoff and Ponstingl, 1991; Corbett et al., 1995). Low cytoplasmic RanGTP levels allow importin α to bind importin β, whereas the high nuclear RanGTP concentration favors importin heterodimer dissociation in the nucleus. Thus, the

RanGTP gradient determines from which compartment the importin heterodimer can transport an NLS substrate.

The export of RNPs from the nucleus to the cytoplasm is probably directed by associated proteins carrying nuclear export signals (NESs; Fischer et al., 1995; Wen et al., 1995) and also requires Ran and its cofactors (for reviews see Gerace, 1995; Görlich and Mattaj, 1996; Izaurralde and Mattaj, 1995). The different classes of RNAs leave the nucleus via distinct routes, each with their own (set of) carrier proteins. For example, the best candidates to mediate export of mRNAs are hnRNP A1 and its relatives (Piñol-Roma and Dreyfuss, 1993), and the cap binding complex (CBC) is involved in export of capped U snRNAs (Izaurralde et al., 1995). Importin α was also recently found to be involved in the export of capped U snRNAs from the nucleus, and release of the RNA molecule into the cytoplasm is mediated by importin β binding to the importin α-RNA complex (Görlich et al., 1996a).

The mechanism of oncogenic activation of the *NUP98-HOXA9* fusion gene is not clear. Preliminary data suggest that NUP98-HOXA9 localizes to the nucleus (J. van Deursen, personal communication), similar to DEK-CAN and SET-CAN (Fornerod et al., 1995). One possibility is that the addition of NUP98 sequences to HOXA9 affects the DNA-binding or transcriptional activity of HOXA9. NUP98 could for instance affect HOXA9-mediated transcription by either contributing a transcriptional activation or repression domain or providing binding sites for proteins imported from the cytosol carrying these functions. Homeobox proteins heterodimerize with other divergent homeodomain proteins, giving yet another possibility for transcriptional deregulation: NUP98-HOXA9 could act as a dominant-negative HOXA9 protein by sequestering co-factors (Borrow et al., 1996). On the other hand, NUP98-HOXA9 may interfere with NPC function. The fusion protein contains the majority of the FG peptide repeats and would therefore be expected to retain the ability to bind importin β, potentially with an adverse effect on nucleocytoplasmic transport.

6 Cellular mechanisms of hematopoietic transformation

The cloning of translocation breakpoints associated with hematopoietic malignancies has identified a variety of proto-oncogenes and their molecular analyses have revealed some principles governing the contribution of translocations and inversions to leukemogenesis. The proteins activated by gene fusions in leukemia often seem to be involved in hematopoietic differentiation. Normal roles in the differentiation program of blood cells are established for AML1, the retinoic acid receptor, and for the homeobox protein PBX1. Fusion proteins derived from these transcription factors block differentiation of hematopoietic progenitors: (i) EVI1, AML1a and the chimeric AML1-EVI1 protein block G-CSF-induced differentiation of the IL-3 dependent 32DCL3

myeloid cell line (Morishita et al., 1992a, 1992b; Tanaka et al., 1995a, 1995b). (ii) RARα fusion proteins block differentiation of promyelocytes in patients and of U937 monoblastic cells to macrophages *in vitro* (Grignani et al., 1993; Redner et al., 1996a; Rogaia et al., 1995; Ruthardt et al., 1996). (iii) E2A-PBX1 strongly retards the differentiation of myeloblasts in primary bone marrow cultures (Kamps and Wright, 1994). It is not clear whether these altered transcription factors block differentiation directly, by inhibiting a programmed pattern of myeloid differentiation genes, or indirectly by preventing a necessary exit from the cell cycle.

Another recurring general mechanism by which fusion proteins contribute to leukemogenesis is the abrogation of growth-factor dependence by inhibition of apoptosis, indicating that some chimeric oncoproteins affect cell survival rather than cell growth: (i) BCR-ABL inhibits apoptosis and renders many different hematopoietic cell lines growth factor independent (for references see section 5.3), and a similar phenotype is found with another altered protein tyrosine kinase, TEL-PDGFRβ, which transforms a murine hematopoietic cell line to IL-3 independence (Carroll et al., 1996). Most likely, these chimeric kinases feed into signal transduction pathways normally activated by a ligand-bound growth factor receptor, thereby bypassing the requirement for the growth factor. (ii) PML-RARα reduces apoptosis upon serum starvation of U937 cells (Grignani et al., 1993; Rogaia et al., 1995). (iii) E2A-HLF protects t(17;19)-positive human leukemia cells and transfected murine pro-B lymphocytes from apoptosis (Inaba et al., 1996).

Paradoxically, some fusion proteins induce apoptosis in immature cells that fail to initiate or complete differentiation. This leads to dysplasia, often accompanied by a compartment enlargement of precursor cells. The enlarged compartment may increase the chances for additional transforming mutations to take place that abrogate the apoptotic response or stimulate proliferation and lead to acute leukemia (Greenberg, 1996; Raza et al., 1996). For example, expression of E2A-PBX1 in transgenic mice induces both enhanced proliferation and apoptosis of T and B cells prior to the development of T-cell lymphomas (Dedera et al., 1993). Also, 32DCL3 cells expressing the *EVI1* zinc finger gene die in the presence of G-CSF without differentiating (Lopingco and Perkins, 1996; Morishita et al., 1992a).

A possible explanation for the success of fusion proteins in transformation may be that the chimeras potentially deregulate functions of both partner proteins, thereby targeting two different pathways in the same cell. The dual functions of fusion proteins are reflected for instance in the combined dominant-negative and gain-of-function activities of AML1 fusions (section 5.1). The requirement for both partners of most chimeric proteins is further demonstrated by the general finding that neither truncation nor overexpression of one of the partners is sufficient to generate the transforming phenotype.

7 Clinical implications

The molecular characterization of fusion transcripts has had a major impact on diagnosis and prognosis of leukemia. First, analysis of the chimeric DNA or RNA in tumors, by Southern blot or reverse transcriptase-polymerase chain reaction (RT-PCR) respectively, can be used for diagnosis in addition to cytogenetics. Second, the fusion transcript is an important target for RT-PCR-based detection of residual tumor cells after treatment and may also assist in early detection of tumors. Third, fusion-specific antibodies can be raised to the junction region which can be used in diagnosis of some translocation-associated diseases, potentially with a sensitivity similar to RT-PCR (Samoszuk et al., 1996; Viswanatha et al., 1996).

The first attempts to treat leukemia using strategies that target the specific molecular abnormality responsible for the disease are becoming a reality. The best example is the widespread use of *all-trans* retinoic acid in the treatment of APL, in which the retinoic acid receptor gene is rearranged. In principle, therapeutic strategies could be applied to interfere with any step between transcription of the oncogene, RNA processing and translation, and function of the fusion protein. The chimeric mRNA can be inactivated either by sequence-specific cleavage of the fusion region by ribozymes or by introduction of antisense oligonucleotides. Both approaches are effective in suppressing the transformed phenotype of cell lines expressing BCR-ABL proteins or AML1-ETO (James et al., 1996 and references therein; Kozu et al., 1996; Matsushita et al., 1995; Sakakura et al., 1994). To inhibit the transforming activity of a specific fusion protein directly, detailed knowledge of its function is necessary. For example, expression of the DNA-binding domain of AML1 inhibited growth and induced differentiation of AML-ETO positive cells (Matsushita et al., 1995; Sakakura et al., 1994). Similar studies show growth inhibition of BCR-ABL positive cells upon co-expression of the oligomerization domain of BCR, or inhibition of the tyrosine kinase activity of ABL (Bergen et al., 1994; Bergen et al., 1995). These approaches will invariably affect the functions of the normal counterparts of the fusion proteins and their usefulness in therapy awaits further investigation.

8 References

Adam, E. J. H., and Adam, S. A. (1994). Identification of cytosolic factors required for nuclear location sequence-mediated binding to the nuclear envelope. J Cell Biol *125*, 547-555.

Adam, S. A., and Gerace, L. (1991). Cytosolic proteins that specifically bind nuclear localization signals are receptors for nuclear import. Cell *66*, 837-847.

Adler, H., and Tkachuk, D. (1996). Leukemic HRX fusion proteins contain novel protein-protein/DNA interaction domains that bind another leukemic factor, the set protein. Blood *88*, 549a.

Ashar, H. R., Fejzo, M. S., Tkachenko, A., Zhou, X., Fletcher, J. A., Weremowicz, S., Morton, C. C., and Chada, K. (1995). Disruption of the architectural factor HMGI-C: DNA-binding AT hook motifs fused in lipomas to distinct transcriptional regulatory domains. Cell *82*, 57-65.

Bedi, A., Zehnbauer, B. A., Barber, J. P., Sharkis, S. J., and Jones, R. J. (1994). Inhibition of apoptosis by BCR-ABL in chronic myeloid leukemia. Blood *83*, 2038-44.

Bergen, R., Connell, Y., Fahmy, B., Kyle, E., and Neckers, L. (1994). Aptameric inhibition of $p210^{bcrabl}$ tyrosine kinase autophosphorylation by oligodeoxynucleotides of defined sequence and backbone structure. Nucleic Acids Res *22*, 2150-2154.

Bergen, R. C., Kyle, E., Connell, Y., and Neckers, L. (1995). Inhibition of protein-kinase activity in intact cells by the aptameric action of oligodeoxynucleotides. Antisense Res Dev *5*, 33-38.

Berlingieri, M. T., Manfioletti, G., Santoro, M., Bandiera, A., Visconti, R., Giancotti, V., and Fusco, A. (1995). Inhibition of HMGI-C protein synthesis suppresses retrovirally induced neoplastic transformation of rat thyroid cells. Mol Cell Biol *15*, 1545-1553.

Bischoff, F. R., Klebe, C., Kretschmer, J., Wittinghofer, A., and Ponstingl, H. (1994). RanGAP1 induces GTPase activity of nuclear ras-related Ran. Proc Natl Acad Sci USA *91*, 2587-2591.

Bischoff, F. R., and Ponstingl, H. (1991). Catalysis of guanine nucleotide exchange on Ran by the mitotic regulator RCC1. Nature *354*, 80-2.

Borrow, J., Shearman, A. M., Stanton, V. P., Jr., Becher, R., Collins, T., Williams, A. J., Dube, I., Katz, F., Kwong, Y. L., Morris, C., Ohyashiki, K., Toyama, K., Rowley, J., and Housman, D. E. (1996). The t(7;11)(p15;p15) translocation in acute myeloid leukaemia fuses the genes for nucleoporin NUP98 and class I homeoprotein HOXA9. Nat Genet *12*, 159-167.

Bustin, M., Lehn, D. A., and Landsman, D. (1990). Structural features of the HMG chromosomal proteins and their genes. Biochim Biophys Acta *1049*, 231-43.

Butturini, A., Arlinghaus, R. B., and Gale, R. P. (1996). BCR/ABL and leukemia. Leukemia Res *20*, 523-529.

Byrd, D. A., Sweet, D. J., Pante, N., Konstantinov, K. N., Guan, T., Saphire, A. C., Mitchell, P. J., Cooper, C. S., Aebi, U., and Gerace, L. (1994). Tpr, a large coiled coil protein whose amino terminus is involved in activation of oncogenic kinases, is localized to the cytoplasmic surface of the nuclear pore complex. J Cell Biol *127*, 1515-1526.

Campbell, M., Li, W., and Arlinghaus, R. B. (1990). $P210^{BCR/ABL}$ is complexed to $P160^{BCR}$ and ph-P53. Oncogene *5*, 773-776.

Carroll, M., MH, T., GF, B., TR, G., and DG, G. (1996). The TEL/platelet-derived growth factor beta receptor (PDGF beta R) fusion in chronic myelomonocytic leukemia is a transforming protein that self-associates and activates PDGF beta R kinase-dependent signaling pathways. Proc Natl Acad Sci USA *93*, 14845-50.

Castilla, L. H., Wijmenga, C., Wang, Q., Stacy, T., Speck, N. A., Eckhaus, M., Marín-Padilla, M., Collins, F. S., Wynshaw-Boris, A., and Liu, P. P. (1996). Failure of embryonic hematopoiesis and lethal hemorrhages in mouse embryos heterozygous for a knocked-in leukemia gene CBFB-MYH11. Cell *87*, 687-696.

Chan, L. C., Karhi, K. K., Rayter, S. I., Heisterkamp, N., Eridani, S., Powles, R., Lawler, S. D., Groffen,

J., Foulkes, J. G., Greaves, M. F., and et al. (1987). A novel abl protein expressed in Philadelphia chromosome positive acute lymphoblastic leukaemia. Nature *325*, 635-7.

Chan, P. K., and Chan, F. Y. (1995). Nucleophosmin/B23 (NPM) oligomer is a major and stable entity in HeLa cells. Biochim Biophys Acta *1262*, 37-42.

Chang, C. P., Shen, W. F., Rozenfeld, S., Lawrence, H. J., Largman, C., and Cleary, M. L. (1995). Pbx proteins display hexapeptide-dependent cooperative DNA binding with a subset of Hox proteins. Genes Dev *9*, 663-674.

Chen, Z., Brand, N. J., Chen, A., Chen, S. J., Tong, J. H., Wang, Z. Y., Waxman, S., and Zelent, A. (1993). Fusion between a novel Kruppel-like zinc finger gene and the retinoic acid receptor-alpha locus due to a variant t(11;17) translocation associated with acute promyelocytic leukaemia. EMBO J *12*, 1161-7.

Chen, Z., Guidez, F., Rousselot, P., Agadir, A., Chen, S. J., Wang, Z. Y., Degos, L., Zelent, A., Waxman, S., and Chomienne, C. (1994). PLZF-RAR alpha fusion proteins generated from the variant t(11;17)(q23;q21) translocation in acute promyelocytic leukemia inhibit ligand-dependent transactivation of wild-type retinoic acid receptors. Proc Natl Acad Sci U S A *91*, 1178-82.

Chi, N. C., Adam, E. J. H., and Adam, S. A. (1995). Sequence and characterization of cytoplasmic nuclear protein import factor p97. J Cell Biol *130*, 265-274.

Chomienne, C., Ballerini, P., Balitrand, N., Daniel, M. T., Fenaux, P., Castaigne, S., and Degos, L. (1990). All-*trans* retinoic acid in acute promyelocytic leukemias. II. In vitro studies: structure-function relationship. Blood *76*, 1710-7.

Clark, S. S., McLaughlin, J., Timmons, M., Pendergast, A. M., Ben-Neriah, Y., Dow, L. W., Crist, W., Rovera, G., Smith, S. D., and Witte, O. N. (1988). Expression of a distinctive *BCR-ABL*oncogene in Ph1-positive acute lymphocytic leukemia (ALL). Science *235*, 85-88.

Cohen, P. (1989). The structure and regulation of protein phosphatases. Annu Rev Biochem *58*, 453-508.

Cook, W. D., Metcalf, D., Nicola, N. A., Burgess, A. W., and Walker, F. (1985). Malignant transformation of a growth factor-dependent myeloid cell line by Abelson virus without evidence of an autocrine mechanism. Cell *41*, 677-83.

Corbett, A. H., Koepp, D. M., Schlenstedt, G., Lee, M. S., Hopper, A. K., and Silver, P. A. (1995). Rnalp, a Ran/TC4 GTPase activating protein, is required for nuclear import. J Cell Biol *130*, 1017-1713.

Corral, J., Forster, A., Thompson, S., Lampert, F., Kaneko, Y., Slater, R., Kroes, W. G., Van der Schoot, C. E., Ludwig, W. D., Karpas, A., and et al. (1993). Acute leukemias of different lineages have similar MLL gene fusions encoding related chimeric proteins resulting from chromosomal translocation. Proc Natl Acad Sci U S A *90*, 8538-42.

Corral, J., Lavenir, I., Impey, H., Warren, A. J., Forster, A., Larson, T. A., Bell, S., McKenzie, A. N., King, G., and Rabbitts, T. H. (1996). An M11-AF9 fusion gene made by homologous recombination causes acute leukemia in chimeric mice: A method to create fusion oncogenes. Cell *85*, 853-861.

Daga, A., Tighe, J. E., and Calabi, F. (1992). Leukaemia/Drosophila homology [letter]. Nature *356*, 484.

Daley, G. Q., and Baltimore, D. (1988). Transformation of an interleukin 3-dependent hematopoietic cell line by the chronic myelogenous leukemia-specific P210bcr/abl protein. Proc Natl Acad Sci U S A *85*, 9312-6.

Daley, G. Q., Van Etten, R., and Baltimore, D. (1990). Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome. Science *247*, 824-830.

De Thé, H., Lavau, C., Marchio, A., Chomienne, C., Degos, L., and Dejean, A. (1991). The PML-RARA fusion mRNA generated by the t(15;17) translocation in acute promyelocytic leukemia encodes a functionally altered RAR. Cell *66*, 675-684.

Dedera, D. A., Waller, E. K., LeBrun, D. P., Sen-Majumdar, A., Stevens, M. E., Barsh, G. S., and Cleary, M. L. (1993). Chimeric homeobox gene E2A-PBX1 induces proliferation, apoptosis, and malignant lymphomas in transgenic mice. Cell *74*, 833-43.

Diekmann, D., Brill, S., Garrett, M. D., Totty, N., Hsuan, J., Monfries, C., Hall, C., Lim, L., and Hall, A.

(1991). Bcr encodes a GTPase-activating protein for p21rac. Nature *351*, 400-2.

Djabali, M., Selleri, L., Parry, P., Bower, M., Young, B. D., and Evans, G. A. (1992). A trithorax-like gene is interrupted by chromosome 11q23 translocations in acute leukaemias [published erratum appears in Nature Genet 1993 Aug;4(4):431]. Nature Genet *2*, 113-118.

Domer, P. H., Fakharzadeh, S. S., Chen, C. S., Jockel, J., Johansen, L., Silverman, G. A., Kersey, J. H., and Korsmeyer, S. J. (1993). Acute mixed-lineage leukemia t(4;11)(q21;q23) generates an MLL-AF4 fusion product. Proc Natl Acad Sci USA *90*, 7884-7888.

Dong, S., Zhu, J., Reid, A., Strutt, P., Guidez, F., Zhong, H. J., Wang, Z. Y., Licht, J., Waxman, S., Chomienne, C., Chen, Z., Zelent, A., and Chen, S. J. (1996). Amino-terminal protein-protein interaction motif (POZ-domain) is responsible for activities of the promyelocytic leukemia zinc finger-retinoic acid receptor-alpha fusion protein. Proc. Natl. Acad. Sci. U.S.A. *93*, 3624-3629.

Downing, J. R., Head, D. R., Raimondi, S. C., Carroll, A. J., Curcio-Brint, A. M., Motroni, T. A., Hulshof, M. G., Pullen, D. J., and Domer, P. H. (1994). The der(11)-encoded MLL/AF-4 fusion transcript is consistently detected in t(4;11)(q21;q23)-containing acute lymphoblastic leukemia. Blood *83*, 330-5.

Doye, V., and Hurt, E. C. (1995). Genetic approaches to nuclear pore structure and function. Trends Genet *11*, 235-241.

Drolet, D. W., Scully, K. M., Simmons, D. M., Wegner, M., Chu, K. T., Swanson, L. W., and Rosenfeld, M. G. (1991). TEF, a transcription factor expressed specifically in the anterior pituitary during embryogenesis, defines a new class of leucine zipper proteins. Genes Dev *5*, 1739-53.

Du, W., Thanos, D., and Maniatis, T. (1993). Mechanisms of transcriptional synergism between distinct virus- inducible enhancer elements. Cell *74*, 887-98.

Dyck, J. A., Maul, G. G., Miller, W. H., Jr., Chen, J. D., Kakizuka, A., and Evans, R. M. (1994). A novel macromolecular structure is a target of the promyelocyte- retinoic acid receptor oncoprotein. Cell *76*, 333-43.

Elefanty, A. G., Hariharan, I. K., and Cory, S. (1990). BCR-ABL, the hallmark of chronic myeloid leukemia in man, induces multiple hematopoietic neoplasms in mice. EMBO J *9*, 1069-1078.

Erickson, P., Gao, J., Chang, K. S., Look, T., Whisenant, E., Raimondi, S., Lasher, R., Trujillo, J., Rowley, J., and Drabkin, H. (1992). Identification of breakpoints in t(8;21) acute myelogenous leukemia and isolation of a fusion transcript, AML1/ETO, with similarity to Drosophila segmentation gene, runt. Blood *80*, 1825-31.

Fabre, E., and Hurt, E. C. (1994). Nuclear transport. Curr Opin Cell Biol *6*, 335-342.

Fischer, U., Huber, J., Boelens, W. C., Mattaj, I. W., and Luhrmann, R. (1995). The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. Cell *82*, 475-485.

Fornerod, M., Boer, J., Van Baal, S., Jaeglé, M., Von Lindern, M., Murti, K. G., Davis, D., Bonten, J., Buijs, A., and Grosveld, G. (1995). Relocation of the carboxyterminal part of CAN from the nuclear envelope to the nucleus as a result of leukemia-specific chromosome rearrangements. Oncogene *10*, 1739-1748.

Fu, G., Grosveld, G., and Markovitz, D. (1997). DEK, an autoantigen involved in a chromosomal translocation in acute myelogenous leukemia, binds to the HIV-2 enhancer. Proc Natl Acad Sci USA *94*, 1811-1815.

Gerace, L. (1995). Nuclear export signals and the fast track to the cytoplasm. Cell *82*, 341-344.

Giancotti, V., Buratti, E., Perissin, L., Zorzet, S., Balmain, A., Portella, G., Fusco, A., and Goodwin, G. H. (1989). Analysis of the HMGI nuclear proteins in mouse neoplastic cells induced by different procedures. Exp Cell Res *184*, 538-545.

Gillard, E. F., and Solomon, E. (1993). Acute promyelocytic leukaemia and the t(15;17) translocation. Semin Cancer Biol *4*, 359-367.

Golub, T. R., Barker, G. F., Bohlander, S. K., Hiebert, S. W., Ward, D. C., Bray-Ward, P., Morgan, E., Raimondi, S. C., Rowley, J. D., and Gilliland, D. G. (1995). Fusion of the TEL gene on 12p13 to the

AML1 gene on 21q22 in acute lymphoblastic leukemia. Proc Natl Acad Sci USA *92*, 4917-4921.

Golub, T. R., Barker, G. F., Lovett, M., and Gilliland, D. G. (1994). Fusion of PDGF receptor á to a novel ets-like gene, tel, in chronic myelomonocytic leukemia with t(5;12) chromosomal translocation. Cell *77*, 307-316.

Golub, T. R., Goga, A., Barker, G. F., Afar, D. E., McLaughlin, J., Bohlander, S. K., Rowley, J. D., Witte, O. N., and Gilliland, D. G. (1996). Oligomerizaton of the ABL tyrosine kinase by the Ets protein TEL in human leukemia. Mol Cell Biol *16*, 4107-4116.

Görlich, D., Kraft, R., Kostka, S., Vogel, F., Hartmann, E., Laskey, R. A., Mattaj, I. W., and Izaurralde, E. (1996a). Importin provides a link between nuclear protein import and U snRNA export. Cell *87*, 21-32.

Görlich, D., and Mattaj, I. (1996). Nucleocytoplasmic transport. Science *271*, 1513-1518.

Görlich, D., Panté, N., Kutay, U., Aebi, U., and Bischoff, F. R. (1996b). Identification of different roles for RanGDP and RanGTP in nuclear protein import. EMBO J *15*, 5584-5594.

Görlich, D., Prehn, S., Laskey, R. A., and Hartmann, E. (1994). Isolation of a protein that is essential for the first step of nuclear protein import. Cell *79*, 767-778.

Görlich, D., Vogel, F., Mills, A. D., Hartmann, E., and Laskey, R. A. (1995). Distinct functions for the two importin subunits in nuclear protein import. Nature *377*, 246-248.

Greco, A., Pierotti, M. A., Bongarzone, I., Pagliardini, S., Lanzi, C., and Della Porta, G. (1992). TRK-T1 is a novel oncogene formed by the fusion of TPR and TRK genes in human papillary thyroid carcinomas. Oncogene *7*, 237-42.

Green, A. (1992). Transcription factors translocations and haematological malignancies. Blood *6*, 118-24.

Greenberg, P. L. (1996). Biologic and clinincal implications of marrow culture studies in the myelodysplastic syndromes. Semin Hematol *33*, 163-175.

Grignani, F., Ferrucci, P. F., Testa, U., Talamo, G., Fagioli, M., Alcalay, M., Mencarelli, A., Grignani, F., Peschle, C., Nicoletti, I., and et al. (1993). The acute promyelocytic leukemia-specific PML-RAR alpha fusion protein inhibits differentiation and promotes survival of myeloid precursor cells. Cell *74*, 423-31.

Grignani, F., Testa, U., Rogaia, D., Ferrucci, P. F., Samoggia, P., Pinto, A., Aldinucci, D., Gelmetti, V., Fagioli, M., Alcalay, M., Seeler, J., Grignani, F., Nicoletti, I., Peschle, C., and Pelicci, P. G. (1996). Effects on differentiation by the promyelocytic leukemia PML/RARα protein depend on the fusion of the PML protein dimerization and RARα DNA binding domains. EMBO J *15*, 4949-4958.

Grisolano, J. L., Wesselschmidt, R. L., Pelicci, P. G., and Ley, T. J. (1997). Altered myeloid development and acute leukemia in transgenic mice expressing PML-RARα under control of cathepsin G regulatory sequences. Blood *89*, 376-387.

Groffen, J., Stephenson, J. R., Heisterkamp, N., de Klein, A., Bartram, C. R., and Grosveld, G. (1984). Philadelphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22. Cell *36*, 93-9.

Groffen, J., Voncken, J. W., Kaartinen, V., Morris, C., and Heisterkamp, N. (1993). Ph-positive leukemia: a transgenic mouse model. Leuk Lymphoma *11 Suppl 1*, 19-24.

Grosschedl, R., Giese, K., and Pagel, J. (1994). HMG domain proteins: architectural elements in the assembly of nucleoprotein structures. Trends Genet *10*, 94-100.

Gu, Y., Nakamura, T., Alder, H., Prasad, R., Canaani, O., Cimino, G., Croce, C. M., and Canaani, E. (1992). The t(4;11) chromosome translocation of human acute leukemias fuses the ALL-1 gene, related to Drosophila trithorax, to the AF-4 gene. Cell *71*, 701-708.

Hariharan, I. K., Adams, J. M., and Cory, S. (1988). bcr-abl oncogene renders myeloid cell line factor independent: potential autocrine mechanism in chronic myeloid leukemia. Oncogene Res *3*, 387-99.

Heisterkamp, N., Jenster, G., ten, H. J., Zovich, D., Pattengale, P. K., and Groffen, J. (1990). Acute leukaemia in bcr/abl transgenic mice. Nature *344*, 251-253.

Henthorn, P., Kiledjian, M., and Kadesch, T. (1990). Two distinct transcription factors that bind the

immunoglobulin enhancer microE5/kappa 2 motif. Science *247*, 467-70.

Hermans, A., Heisterkamp, N., von Linden, M., Van Baal, S., Meijer, D., Van der Plas, D., Wiedemann, L. M., Groffen, J., Bootsma, D., and Grosveld, G. (1987). Unique fusion of bcr and c-abl genes in Philadelphia chromosome positive acute lymphoblastic leukemia. Cell *51*, 33-40.

Hess, J., Yu, B., Hanson, R., Li, B., Brown, G., and Korsmeyer, S. (1996). The mixed lineage leukemia gene (MLL) is requied for maintenance of hox gene expression and for normal hematopoiesis. Blood *88*, 48a.

Hiebert, S. W., Sun, W., Davis, J. N., Golub, T., Shurtleff, S., Buijs, A., Downing, J. R., Grosveld, G., Roussel, M. F., Gilliland, D. G., Lenny, N., and Meyers, S. (1996). The t(12;21) translocation converts AML-1B from an activator to a repressor of transcription. Mol Cell Biol *16*, 1349-1355.

Huang, M. E., Ye, Y. C., Chen, S. R., Chai, J. R., Lu, J. X., Zhoa, L., Gu, L. J., and Wang, Z. Y. (1988). Use of all-*trans* retinoic acid in the treatment of acute promyelocytic leukemia. Blood *72*, 567-72.

Hunger, S. P., Brown, R., and Cleary, M. L. (1994). DNA-binding and transcriptional regulatory properties of hepatic leukemia factor (HLF) and the t(17;19) acute lymphoblastic leukemia chimera E2A-HLF. Mol Cell Biol *14*, 5986-5996.

Hunger, S. P., Ohyashiki, K., Toyama, K., and Cleary, M. L. (1992). Hlf, a novel hepatic bZIP protein, shows altered DNA-binding properties following fusion to E2A in t(17;19) acute lymphoblastic leukemia. Genes Dev *6*, 1608-20.

Iida, S., Seto, M., Yamamoto, K., Komatsu, H., Tojo, A., Asano, S., Kamada, N., Ariyoshi, Y., Takahashi, T., and Ueda, R. (1993). MLLT3 gene on 9p22 involved in t(9;11) leukemia encodes a serine/proline rich protein homologous to MLLT1 on 19p13. Oncogene *8*, 3085-92.

Inaba, T., Inukai, T., Yoshihara, T., Seyschab, H., Ashmun, R. A., Canman, C. E., Laken, S. J., Kastan, M. B., and Look, A. T. (1996). Reversal of apoptosis by the leukaemia-associated E2A-HLF chimaeric transcription factor. Nature *382*, 541-544.

Inaba, T., Roberts, W. M., Shapiro, L. H., Jolly, K. W., Raimondi, S. C., Smith, S. D., and Look, A. T. (1992). Fusion of the leucine zipper gene HLF to the E2A gene in human acute B- lineage leukemia. Science *257*, 531-4.

Inaba, T., Shapiro, L. H., Funabiki, T., Sinclair, A. E., Jones, B. G., Ashmun, R. A., and Look, A. T. (1994). DNA-binding specificity and trans-activating potential of the leukemia- associated E2A-hepatic leukemia factor fusion protein. Mol Cell Biol *14*, 3403-13.

Inukai, T., Inaba, T., Ikushima, S., and Look, A. (1996). Anti-apoptotic effects of E2A-HLF: evidence for mechanisms involving both gain and loss of function. Blood *88*, 552a.

Iovine, M. K., Watkins, J. L., and Wente, S. R. (1995). The GLFG repetitive region of the nucleoporin Nup116p interacts with Kap95p, an essential yeast nuclear import factor. J Cell Biol *131*, 1699-1713.

Izaurralde, E., Lewis, J., Gamberi, C., Jarmolowski, A., McGuigan, C., and Mattaj, I. W. (1995). A cap-binding protein complex mediating U snRNA export. Nature *376*, 709-712.

Izaurralde, E., and Mattaj, I. W. (1995). RNA export. Cell *81*, 153-159.

James, H., Mills, K., and Gibson, I. (1996). Investigating and improving the specificity of ribozymes directed against the *bcr-abl* translocation. Leukemia *10*, 1054-1064.

Jansen, J. H., Mahfoudi, A., Rambaud, S., Lavau, C., Wahli, W., and Dejean, A. (1995). Multimeric complexes of the PML-retinoic acid receptor alpha fusion protein in acute promyelocytic leukemia cells and interference with retinoid and peroxisome-proliferator signaling pathways. Proc Natl Acad Sci U S A *92*, 7401-7405.

Jousset, C., Carron, C., Boureux, A., Quang, C. T., Oury, C., Dusanter-Fourt, I., Charon, M., Levin, J., Bernard, O., and Ghysdael, J. (1997). A domain of TEL conserved in a subset of ETS proteins defines a specific oligomerization interface essential to the mitogenic properties of the TEL-PDGFRβ oncoprotein. EMBO J *16*, 69-82.

Kakizuka, A., Miller, W. H., Umesono, K., Warrell, R. P., Frankel, S. R., Murty, V. V. V. S., Dmitrovsky,

E., and Evans, R. M. (1991). Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RARA with a novel putative transcription factor, PML. Cell *66*, 663-674.

Kamps, M. P., and Baltimore, D. (1993). E2A-Pbx1, the t(1;19) translocation protein of human pre-B-cell acute lymphocytic leukemia, causes acute myeloid leukemia in mice. Mol Cell Biol *13*, 351-7.

Kamps, M. P., Look, A. T., and Baltimore, D. (1991). The human t(1;19) translocation in pre-B ALL produces multiple nuclear E2A-Pbx1 fusion proteins with differing transforming potentials. Genes Dev *5*, 358-368.

Kamps, M. P., Murre, C., Sun, X. H., and Baltimore, D. (1990). A new homeobox gene contributes the DNA binding domain of the t(1;19) translocation protein in pre-B ALL. Cell *60*, 547-555.

Kamps, M. P., and Wright, D. D. (1994). Oncoprotein E2A-Pbx1 immortalizes a myeloid progenitor in primary marrow cultures without abrogating its factor-dependence. Oncogene *9*, 3159-3166.

Kania, M. A., Bonner, A. S., Duffy, J. B., and Gergen, J. P. (1990). The Drosophila segmentation gene runt encodes a novel nuclear regulatory protein that is also expressed in the developing nervous system. Genes Dev *4*, 1701-13.

Kastner, P., Grondona, J. M., Mark, M., Gansmuller, A., LeMeur, M., Decimo, D., Vonesch, J. L., Dolle, P., and Chambon, P. (1994). Genetic analysis of RXR alpha developmental function: convergence of RXR and RAR signaling pathways in heart and eye morphogenesis. Cell *78*, 987-1003.

Kastner, P., Perez, A., Lutz, Y., Rochette-Egly, C., Gaub, M., B, D., Lanotte, M., Berger, R., and Chambon, P. (1992). Structure, localization and transcriptional properties of two classes of retinoic acid receptor à fusion proteins in acute promyelocytic leukemia (APL): structural similarities with a new family of oncoproteins. EMBO J *11*, 629-642.

Kazmierczak, B., Hennig, Y., Wanschura, S., Rogalla, P., Bartnitzke, S., Van de Ven, W., and Bullerdiek, J. (1995). Description of a novel fusion transcript between HMGI-C, a gene encoding for a member of the high mobility group proteins, and the mitochondrial aldehyde dehydrogenase gene. Cancer Res *55*, 6038-6039.

Kelliher, M., Knott, A., McLaughlin, J., Witte, O. N., and Rosenberg, N. (1991). Differences in oncogenic potency but not target cell specificity distinguish the two forms of the BCR/ABL oncogene. Mol Cell Biol *11*, 4710-4716.

Kelliher, M. A., McLaughlin, J., Witte, O. N., and Rosenberg, N. (1990). Induction of a chronic myelogenous leukemia-like syndrome in mice with v-abl and BCR/ABL [published erratum appears in Proc Natl Acad Sci U S A 1990 Nov;87(22):9072]. Proc Natl Acad Sci U S A *87*, 6649-53.

Kellogg, D. R., Kikuchi, A., Fujii-Nakata, T., Turck, C. W., and Murray, A. W. (1995). Members of the NAP/SET family of proteins interact specifically with B-type cyclins. J Cell Biol *130*, 661-673.

Kobayashi, H., Montgomery, K. T., Bohlander, S. K., Adra, C. N., Lim, B. L., Kucherlapati, R. S., Donis-Keller, H., Holt, M. S., Le Beau, M. M., and Rowley, J. D. (1994). Fluorescence in situ hybridization mapping of translocations and deletions involving the short arm of human chromosome 12 in malignant hematologic diseases. Blood *84*, 3473-3482.

Koken, M., Reid, A., Quignon, F., Dong, S., Chen, Z., Strutt, P., Licht, J., Waxman, S., de Thé, H., and Zelent, A. (1996). Products of the PML and PLZF genes translocated with the RAR alpha locus in acute promyelocytic leukemia co-localize in the nucleus and interact with each other in vivo. Blood *88*, 553a.

Koken, M. H., Puvion-Dutilleul, F., Guillemin, M. C., Viron, A., Linares-Cruz, G., Stuurman, N., de Jong, L., Szostecki, C., Calvo, F., Chomienne, C., and et al. (1994). The t(15;17) translocation alters a nuclear body in a retinoic acid- reversible fashion. EMBO J *13*, 1073-83.

Konopka, J. B., Watanabe, S. M., and Witte, O. N. (1984). An alteration of the human c-abl protein in K562 leukemia cells unmasks associated tyrosine kinase activity. Cell *37*, 1035-1042.

Kozu, T., Sueoka, E., Okabe, S., Sueoka, N., Komori, A., and Fujiki, H. (1996). Designing of chimeric DNA/RNA hammerhead ribozymes to be targeted against AML1/MTG8 mRNA. J Cancer Res Clin Oncol *122*, 254-256.

Kraemer, D., Wozniak, R. W., Blobel, G., and Radu, A. (1994). The human can protein, a putative oncogene product associated with myeloid leukemogenesis, is a nuclear pore complex protein that faces the cytoplasm. Proc Natl Acad Sci USA *91*, 1519-1523.

Kraemer, D. M., Strambio-de-Castillia, C., Blobel, G., and Rout, M. P. (1995). The essential yeast nucleoporin NUP159 is located on the cytoplasmic side of the nuclear pore complex and serves in karyopherin-mediated binding of transport substrate. J Biol Chem *270*, 19017-19021.

Kurzrock, R., Gutterman, J. U., and Talpaz, M. (1988). The molecular genetics of Philadelphia chromosome-positive leukemias. N Engl J Med *319*, 990-998.

Kurzrock, R., Shtalrid, M., Romero, P., Kloetzer, W. S., Talpas, M., Trujillo, J. M., Blick, M., Beran, M., and Gutterman, J. U. (1987). A novel c-abl protein product in Philadelphia-positive acute lymphoblastic leukaemia. Nature *325*, 631-5.

Li, M., Makkinje, A., and Damuni, Z. (1996). The myeloid leukemia-associated protein SET is a potent inhibitor of protein phosphatase 2A. J Biol Chem *271*, 11059-11062.

Liang, T. J., Reid, A. E., Xavier, R., Cardiff, R. D., and Wang, T. C. (1996). Transgenic expression of tpr-met oncogene leads to development of mammary hyperplasia and tumors. J Clin Invest *97*, 2872-2877.

Licht, J. D., Shaknovich, R., English, M. A., Melnick, A., Li, J. Y., Reddy, J. C., Dong, S., Chen, S. J., Zelent, A., and Waxman, S. (1996). Reduced and altered DNA-binding and transcriptional properties of the PLZF-retinoic acid receptor-alpha chimera generated in t(11;17)- associated acute promyelocytic leukemia. Oncogene *12*, 323-336.

Liu, P., Siedel, N., Bodine, D., Speck, N., Tarle, S., and Collins, F. S. (1994). Acute myeloid leukemia with inv(16) produces a chimeric transcription factor with a myosin heavy chain tail. Cold Spring Harbor Symp Quant Biol *59*, 547-553.

Liu, P., Tarlé, S. A., Hajra, A., Claxton, D. F., Marlton, P., Freedman, M., Siciliano, M. J., and Collins, F. S. (1993). Fusion between transcription factor CBF beta/PEBP2 beta and a myosin heavy chain in acute myeloid leukemia. Science *261*, 1041-4.

Lo Coco, F., Mandelli, F., Breccia, M., Annino, L., Gugiielmi, C., Petti, M. C., Testi, A. M., Alimena, G., Croce, C. M., Canaani, E., and Cimino, G. (1993). Southern blot analysis of *ALL-1* rearrangements at chromosome 11q23 in acute leukemia. Cancer Res *53*, 3800-3803.

Lopingco, M. C., and Perkins, A. S. (1996). Molecular analysis of Evi1, a zinc finger oncogene involved in myeloid leukemia. Curr. Top. Microbiol. Immunol. *211*, 211-222.

Lovering, R., Hanson, I. M., Borden, K. L., Martin, S., NJ, O. R., Evan, G. I., Rahman, D., Pappin, D. J., Trowsdale, J., and Freemont, P. S. (1993). Identification and preliminary characterization of a protein motif related to the zinc finger. Proc Natl Acad Sci U S A *90*, 2112-6.

Ma, Q., Alder, H., Nelson, K. K., Chatterjee, D., Gu, Y., Nakamura, T., Canaani, E., Croce, C. M., Siracusa, L. D., and Buchberg, A. M. (1993). Analysis of the murine *ALL-1* gene reveals conserved domains with human *ALL-1* and identifies a motif shared with DNA methyltransferases. Proc Natl Acad Sci USA *90*, 6350-6354.

Mandanas, R. A., Boswell, H. S., Lu, L., and Leibowitz, D. (1992). BCR/ABL confers growth factor independence upon a murine myeloid cell line. Leukemia *6*, 796-800.

Maru, Y., and Witte, O. N. (1991). The BCR gene encodes a novel serine/threonine kinase activity within a single exon. Cell *67*, 459-68.

Matsushita, H., Kobayashi, H., Mori, S., Kizaki, M., and Ikeda, Y. (1995). Ribozymes cleave the AML1/MTG8 fusion transcript and inhibit proliferation of leukemic cells with t(8;21). Biochem Biophys Res Commun *215*, 431-437.

McGahon, A., Bissonnette, R., Schmitt, M., Cotter, K. M., Green, D. R., and Cotter, T. G. (1994). BCR-ABL maintains resistance of chronic myelogenous leukemia cells to apoptotic cell death. Blood *83*, 1179-87.

McGinnis, W., Garber, R. L., Wirz, J., Kuroiwa, A., and Gehring, W. J. (1984). A homologous protein-

coding sequence in Drosophila homeotic genes and its conservation in other metazoans. Cell *37*, 403-408.

McWhirter, J. R., Galasso, D. L., and Wang, J. Y. (1993). A coiled-coil oligomerization domain of Bcr is essential for the transforming function of Bcr-Abl oncoproteins. Mol Cell Biol *13*, 7587-95.

Melchior, F., Paschal, B., Evans, J., and Gerace, L. (1993). Inhibition of nuclear protein import by nonhydrolyzable analogues of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor [published erratum appears in J Cell Biol 124:217, 1994]. J Cell Biol *123*, 1649-1659.

Meyers, S., Downing, J. R., and Hiebert, S. W. (1993). Identification of AML-1 and the (8;21) translocation protein (AML-1/ETO) as sequence-specific DNA-binding proteins: The *runt* homology domain is required for DNA binding and protein-protein interactions. Mol Cell Biol *13*, 6336-6345.

Meyers, S., Lenny, N., and Hiebert, S. W. (1995). The t(8;21) fusion protein interferes with AML-1B-dependent transcriptional activation. Mol Cell Biol *15*, 1974-1982.

Mitani, K., Ogawa, S., Tanaka, T., Miyoshi, H., Kurokawa, M., Mano, H., Yazaki, Y., Ohki, M., and Hirai, H. (1994). Generation of the *AML1-EVI-1* fusion gene in the t(3;21)(q26;q22) causes blastic crisis in chronic myelocytic leukemia. EMBO J *13*, 504-510.

Miyoshi, H., Kozu, T., Shimizu, K., Enomoto, K., Maseki, N., Kaneko, Y., Kamada, N., and Ohki, M. (1993). The t(8;21) translocation in acute myeloid leukemia results in production of an *AML1-MTG8* fusion transcript. EMBO J *12*, 2715-2721.

Miyoshi, H., Ohira, M., Shimizu, K., Mitani, K., Hirai, H., Imai, T., Yokoyama, K., Soeda, E., and Ohki, M. (1995). Alternative splicing and genomic structure of the AML1 gene involved in acute myeloid leukemia. Nucleic Acids Res *23*, 2762-2769.

Monica, K., LeBrun, D. P., Dedera, D. A., Brown, R., and Cleary, M. L. (1994). Transformation properties of the E2a-Pbx1 chimeric oncoprotein: fusion with E2a is essential, but the Pbx1 homeodomain is dispensable. Mol. Cell. Biol. *14*, 8304-14.

Moore, M. S., and Blobel, G. (1993). The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. Nature *365*, 661-663.

Morishita, K., Parganas, E., Matsugi, T., and Ihle, J. N. (1992a). Expression of the Evi-1 zinc finger gene in 32Dc13 myeloid cells blocks granulocytic differentiation in response to granulocyte colony-stimulating factor. Mol Cell Biol *12*, 183-9.

Morishita, K., Parganas, E., Willman, C. L., Whittaker, M. H., Drabkin, H., Oval, J., Taetle, R., Valentine, M. B., and Ihle, J. N. (1992b). Activation of *EVI1* gene expression in human acute myelogenous leukemias by translocations spanning 300-400 kilobases on chromosome band 3q26. Proc Natl Acad Sci USA *89*, 3937-3941.

Moroianu, J., Blobel, G., and Radu, A. (1995a). Previously identified protein of uncertain function is karyopherin α and together with karyopherin β docks import substrate at nuclear pore complexes. Proc Natl Acad Sci USA *92*, 2008-2011.

Moroianu, J., Hijikata, M., Blobel, G., and Radu, A. (1995b). Mammalian karyopherin α1β and α2β heterodimers: α1 or α2 subunit binds nuclear localization signal and β subunit interacts with peptide repeat-containing nucleoporins. Proc Natl Acad Sci USA *92*, 6532-6536.

Morrissey, J., Tkachuk, D. C., Milatovich, A., Francke, U., Link, M., and Cleary, M. L. (1993). A serine/proline-rich protein is fused to HRX in t(4;11) acute leukemias. Blood *81*, 1124-1131.

Muller, A. J., Pendergast, A. M., Havlik, M. H., Puil, L., Pawson, T., and Witte, O. N. (1992). A limited set of SH2 domains bind BCR through a high-affinity phosphotyrosine-independent interaction. Mol Cell Biol *12*, 5087-5093.

Muller, A. J., Young, J. C., Pendergast, A., Pondel, M., Landau, N. R., Littman, D. R., and Witte, O. N. (1991). BCR first exon sequences specifically activate the BCR/ABL tyrosine kinase oncogene of Philadelphia chromosome-positive human leukemias. Mol Cell Biol *11*, 1785-1792.

Mumby, M. C., and Walter, G. (1993). Protein serine/threonine phosphatases: structure, regulation, and

functions in cell growth. Physiol Rev *73*, 673-699.

Murre, C., McCaw, P. S., and Baltimore, D. (1989). A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. Cell *56*, 777-83.

Nagata, K., Kawase, H., Handa, H., Yano, K., Yamasaki, M., Ishimi, Y., Okuda, A., Kikuchi, A., and Matsumoto, K. (1995). Replication factor encoded by a putative oncogene, *set*, associated with myeloid leukemogenesis. Proc Natl Acad Sci USA *92*, 4279-4283.

Nakamura, T., Alder, H., Gu, Y., Prasad, R., Canaani, O., Kamada, N., Gale, R. P., Lange, B., Crist, W. M., Nowell, P. C., Croce, C. M., and Canaani, E. (1993). Genes on chromosome 4, 9 and 19 involved in 11q23 abnormalities in acute leukemia share homology and/or common motifs. Proc Natl Acad Sci USA *90*, 4631-4635.

Nakamura, T., Largaespada, D. A., Lee, M. P., Johnson, L. A., Ohyashiki, K., Toyama, K., Chen, S. J., Willman, C. L., Chen, I. M., Feinberg, A. P., Jenkins, N. A., Copeland, N. G., and Shaughnessy, J. D., Jr. (1996a). Fusion of the nucleoporin gene NUP98 to HOXA9 by the chromosome translocation t(7;11)(p15;p15) in human myeloid leukaemia. Nat Genet *12*, 154-158.

Nakamura, T., Largaespada, D. A., Shaughnessy, J. D., Jr., Jenkins, N. A., and Copeland, N. G. (1996b). Cooperative activation of Hoxa and Pbx1-related genes in murine myeloid leukaemias. Nature Genet. *12*, 149-153.

Nehrbass, U., and Blobel, G. (1996). Role of the nuclear transport factor p10 in nuclear import. Science *272*, 120-122.

Nelson, C., LP, S., A, M., E, F., and WJ, R. (1990). Pan: a transcriptional regulator that binds chymotrypsin, insulin, and AP-4 enhancer motifs. Genes Dev *4*, 1035-43.

Nourse, J., Mellentin, J. D., Galili, N., Wilkinson, J., Stanbridge, E., Smith, S. D., and Cleary, M. L. (1990). Chromosomal translocation t(1;19) results in synthesis of a homeobox fusion mRNA that codes for a potential chimeric transcription factor. Cell *60*, 535-545.

Nucifora, G., Begy, C. R., Erickson, P., Drabkin, H. A., and Rowley, J. D. (1993). The 3;21 translocation in myelodysplasia results in a fusion transcript between the AML1 gene and the gene for EAP, a highly conserved protein associated with the Epstein-Barr virus small RNA EBER 1. Proc Natl Acad Sci U S A *90*, 7784-8.

Nucifora, G., Begy, C. R., Kobayashi, H., Roulston, D., Claxton, D., Pedersen-Bjergaard, J., Parganas, E., Ihle, J. N., and Rowley, J. D. (1994). Consistent intergenic splicing and production of multiple transcripts between AML1 at 21q22 and unrelated genes at 3q26 in (3;21)(q26;q22) translocations. Proc Natl Acad Sci U S A *91*, 4004-8.

Okuda, T., Van Deursen, J., Hiebert, S. W., Grosveld, G., and Downing, J. R. (1996a). AML1, the target of multiple choromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. Cell *84*, 321-330.

Okuda, T., Van Deursen, J., Hiebert, S., and Grosveld, G. (1996b). Genetic analysis of AML-1 function in normal and leukemic hematopoiesis. Blood *88*, 290a.

Oñate, S. A., Prendergast, P., Wagner, J. P., Nissen, M., Reeves, R., Pettijohn, D. E., and Edwards, D. P. (1994). The DNA-bending protein HMG-1 enhances progesterone receptor binding to its target DNA sequences. Mol Cell Biol *14*, 3376-91.

O'Neill, E. M., Rebay, I., Tjian, R., and Rubin, G. M. (1994). The activities of two ets-related transcription factors required for Drosophila eye development are modulated by the ras/MAPK pathway. Cell *78*, 137-147.

Pandolfi, P. P., Grignani, F., Alcalay, M., Mencarelli, A., Biondi, A., LoCoco, F., Grignani, F., and Pelicci, P. G. (1991). Structure and origin of the acute promyelocytic leukemia myl/RAR alpha cDNA and characterization of its retinoid-binding and transactivation properties. Oncogene *6*, 1285-92.

Panté, N., and Aebi, U. (1994). Toward the molecular details of the nuclear pore complex. J Struct Biol *113*, 179-189.

Papadopoulos, P., Ridge, S. A., Boucher, C. A., Stocking, C., and Wiedemann, L. M. (1995). The novel

activation of ABL by fusion to an ets-related gene. Cancer Res *55*, 34-38.

Patel, U. A., Bandeira, A., Manfioletti, G., Giancotti, V., Chau, K.-Y., and Crane-Robinson, C. (1994). Expression and cDNA cloning of human HMGI-C phosphoprotein. Biochem Biophys Res Commun *201*, 63-70.

Paull, T. T., Haykinson, M. J., and Johnson, R. C. (1993). The nonspecific DNA-binding and -bending proteins HMG1 and HMG2 promote the assembly of complex nucleoprotein structures. Genes Dev *7*, 1521-34.

Pendergast, A. M., Quilliam, L. A., Cripe, L. D., Bassing, C. H., Dai, Z., Li, N., Batzer, A., Rabun, K. M., Der, C. J., Schlessinger, J., and Gishizky, M. L. (1993). BCR-ABL-induced oncogenesis is mediated by direct interaction with the SH2 domain of the GRB-2 adaptor protein. Cell *75*, 175-185.

Perez, A., Kastner, P., Sethi, S., Lutz, Y., Reibel, C., and Chambon, P. (1993). PMLRAR homodimers: distinct DNA binding properties and heteromeric interactions with RXR. EMBO J *12*, 3171-82.

Pil, P. M., Chow, C. S., and Lippard, S. J. (1993). High-mobility-group 1 protein mediates DNA bending as determined by ring closures. Proc Natl Acad Sci U S A *90*, 9465-9.

Piñol-Roma, S., and Dreyfuss, G. (1993). hnRNP proteins: localization and transport between the nucleus and the cytoplasm. Trends Cell Biol *3*, 151-155.

Rabbitts, T. H. (1994). Chromosome translocations in human cancer. Nature *372*, 143-149.

Radu, A., Blobel, G., and Moore, M. S. (1995a). Identification of a protein complex that is required for nuclear protein import and mediates docking of import substrate to distinct nucleoporins. Proc Natl Acad Sci USA *92*, 1769-1773.

Radu, A., Moore, M. S., and Blobel, G. (1995b). The peptide repeat domain of nucleoporin Nup98 functions as a docking site in transport across the nuclear pore complex. Cell *81*, 215-222.

Ram, T. G., Reeves, R., and Hosick, H. L. (1993). Elevated high mobility group-I(Y) gene expression is associated with progressive transformation of mouse mammary epithelial cells. Cancer Res *53*, 2655-2660.

Raza, A., Mundle, S., Shetty, V., Alvi, S., Chopra, H., Span, L., Parcharidou, A., Dar, S., Venugopal, P., Borok, R., Gezer, S., Showel, J., Loew, J., Robin, E., Rifkin, S., Alston, D., Hernandez, B., Shah, R., Kaizer, H., Gregory, S., and Preisler, H. (1996). A paradigm shift in myelodysplastic syndromes. Leukemia *10*, 1648-1652.

Reddy, B. A., Etkin, L. D., and Freemont, P. S. (1992). A novel zinc finger coiled-coil domain in a family of nuclear proteins. Trends Biochem Sci *17*, 344-345.

Redner, R., Rush, E., and Pollock, S. (1996a). Forced expression of the NPM-RAR fusion gene inhibits differentiation of U937 cells. Blood *88*, 551a.

Redner, R. L., Rush, E. A., Faas, S., Rudert, W. A., and Corey, S. J. (1996b). The t(5;17) variant of acute promyelocytic leukemia expresses a nucleophosmin-retinoic acid receptor fusion. Blood *87*, 882-886.

Reeves, R., and Nissen, M. S. (1990). The A.T-DNA-binding domain of mammalian high mobility group I chromosomal proteins. A novel peptide motif for recognizing DNA structure. J Biol Chem *265*, 8573-82.

Rexach, M., and Blobel, G. (1995). Protein import into nuclei: Association and dissociation reactions involving transport substrate, transport factors, and nucleoporins. Cell *83*, 683-692.

Ringold, S., Libermann, T., and Golub, T. (1996). Functional characterization of the leukemogenic ETS protein TEL evidence for transcriptional repression. Blood *88*, 554a.

Rodrigues, G. A., and Park, M. (1993). Dimerization mediated through a leucine zipper activates the oncogenic potential of the met receptor tyrosine kinase. Mol Cell Biol *13*, 6711-22.

Rogaia, D., Grignani, F., Grignani, F., Nicoletti, I., and Pelicci, P. G. (1995). The acute promyelocytic leukemia-specific PML/RAR alpha fusion protein reduces the frequency of commitment to apoptosis upon growth factor deprivation of GM-CSF-dependent myeloid cells. Leukemia *9*, 1467-1472.

Romana, S. P., Machauffe, M., Le Coniat, M., Chumakov, I., Le Paslier, D., Berger, R., and Bernard, O.

A. (1995). The t(12;21) of acute lymphoblastic leukemia results in a tel-AML1 gene fusion. Blood *85*, 3662-3670.

Rout, M. P., and Wente, S. R. (1994). Pores for thought: Nuclear pore complex proteins. Trends Biochem Sci *4*, 357-363.

Rowley, J. D. (1992). The der(11) chromosome contains the critical breakpoint junction in the 4;11, 9;11, and 11;19 translocations in acute leukemia. Genes Chromosom Cancer *5*, 264-6.

Rowley, J. D., Golomb, H. M., and Dougherty, C. (1977). 15/17 translocation, a consistent chromosomal change in acute promyelocytic leukaemia [letter]. Lancet *1*, 549-50.

Rowley, J. D., and Potter, D. (1976). Chromosomal banding patterns in acute nonlymphocytic leukemia. Blood *47*, 705-721.

Rubnitz, J. E., Behm, F. G., and Downing, J. R. (1996). 11q23 rearrangements in acute leukemia. Leukemia *10*, 74-82.

Rubnitz, J. E., Morrissey, J., Savage, P. A., and Cleary, M. L. (1994). ENL, the gene fused with HRX in t(11;19) leukemias, encodes a nuclear protein with transcriptional activation potential in lymphoid and myeloid cells. Blood *84*, 1747-1752.

Ruthardt, M., Testa, U., Nervi, C., Riganelli, D., Ferrucci, P. F., grignani, F., Alcalay, M., Puccetti, E., Grignani, F., Peschle, C., Hoetzer, D., and Pelicci, P. (1996). Genetic determination of retinoic acid response in acute promyelocytic leukemia. Blood *88*, 551a.

Sakakura, C., Yamaguchi-Iwai, Y., Satake, M., Bae, S. C., Takahashi, A., Ogawa, E., Hagiwara, A., Takahashi, T., Murakami, A., Makino, K., and et al. (1994). Growth inhibition and induction of differentiation of t(8;21) acute myeloid leukemia cells by the DNA-binding domain of PEBP2 and the AML1/MTG8(ETO)-specific antisense oligonucleotide. Proc Natl Acad Sci U S A *91*, 11723-11727.

Samoszuk, M., Sallash, G., tynan, W., and Jr., W. M. (1996). Utility of pml (pg-m3) monoclonal antibody in the evaluation of acute promyelocytic leukemia (APL). Blood *88*, 365a.

Sawyers, C. L. (1997). Molecular genetics of acute leukaemia. Lancet *349*, 196-200.

Sawyers, C. L., Denny, C. T., and Witte, O. N. (1991). Leukemia and the disruption of normal hematopoiesis. Cell *64*, 337-50.

Sawyers, C. L., McLaughlin, J., Goga, A., Havlik, M., and Witte, O. N. (1994). The nuclear tyrosine kinase c-ABL negatively regulates cell growth. Cell, 121-131.

Schichman, S. A., Canaani, E., and Croce, C. M. (1995). Self-fusion of the ALL1 gene. A new genetic mechanism for acute leukemia. Jama *273*, 571-576.

Schoenberg Fejzo, M., Yoon, S.-J., Montgomery, K., Rein, M. S., Weremowicz, S., Krauter, K. S., Dorman, T. E., Fletcher, J. A., Mao, J., Moir, D. T., Kucherlapati, R. S., and Morton, C. C. (1995). Identification of a YAC spanning the translocation breakpoints in uterine leiomyomata, pulmonary chondroid hamartoma and lipoma: physical mapping of the 12q14-q15 breakpoint region in uterine leiomyomata. Genomics *26*, 265-271.

Schoenmakers, E. F., Wanschura, S., Mols, R., Bullerdiek, J., Van den Berghe, H., and Van de Ven, W. J. (1995). Recurrent rearrangements in the high mobility group protein gene, HMGI- C, in benign mesenchymal tumours. Nat Genet *10*, 436-444.

Shenolikar, S., and Nairn, A. C. (1991). Protein phosphatases: recent progress. Adv Second Messenger Phosphoprotein Res *23*, 1-121.

Shtivelman, E., Lifshitz, B., Gale, R. P., and Canaani, E. (1985). Fused transcript of abl and bcr genes in chronic myelogenous leukaemia. Nature *315*, 550-554.

Sirard, C., Laneuville, P., and Dick, J. E. (1994). Expression of bcr-abl abrogates factor-dependent growth of human hematopoietic M07E cells by an autocrine mechanism. Blood *83*, 1575-85.

Soekarman, D., Von Lindern, M., Daenen, S., de Jong, B., Fonatsch, C., Heinze, B., Bartram, C., Hagemeijer, A., and Grosveld, G. (1992). The translocation (6;9)(p23;q34) shows consistent rearrangement of two genes and defines a myeloproliferative disorder with specific clinical features. Blood *79*, 1-8.

Soman, N. R., Correa, P., Ruiz, B. A., and Wogan, G. N. (1991). The TPR-MET oncogenic rearrangement is present and expressed in human gastric carcinoma and precursor lesions. Proc Natl Acad Sci U S A *88*, 4892-6.

Tanaka, T., Mitani, K., Kurokawa, M., Ogawa, S., Tanaka, K., Nishida, J., Yazaki, Y., Shibata, Y., and Hirai, H. (1995a). Dual functions of the AML1/Evi-1 chimeric protein in the mechanism of leukemogenesis in t(3;21) leukemias. Mol Cell Biol *15*, 2383-2392.

Tanaka, T., Tanaka, K., Ogawa, S., Kurokawa, M., Mitani, K., Nishida, J., Shibata, Y., Yazaki, Y., and Hirai, H. (1995b). An acute myeloid leukemia gene, AML1, regulates hemopoietic myeloid cell differentiation and transcriptional activation antagonistically by two alternative spliced forms. EMBO J *14*, 341-350.

Thanos, D., and Maniatis, T. (1992). The high mobility group protein HMGI(Y) is required for NF-κB-dependent virus induction of the human IFN-β gene. Cell *71*, 777-789.

Thirman, M. J., Gill, H. J., Burnett, R. C., Mbangkollo, D., McCabe, N. R., Kobayashi, H., Ziemin-van der Poel, S., Kaneko, Y., Morgan, R., Sandberg, A. A., and et al. (1993). Rearrangement of the MLL gene in acute lymphoblastic and acute myeloid leukemias with 11q23 chromosomal translocations [see comments]. N Engl J Med *329*, 909-14.

Tkachuk, D. C., Kohler, S., and Cleary, M. L. (1992). Involvement of a homolog of Drosophila trithorax by 11q23 chromosomal translocations in acute leukemias. Cell *71*, 691-700.

Van Dijk, M. A., Voorhoeve, P. M., and Murre, C. (1993). Pbx1 is converted into a transcriptional activator upon acquiring the N- terminal region of E2A in pre-B-cell acute lymphoblastoid leukemia. Proc Natl Acad Sci U S A *90*, 6061-5.

Van Etten, R. A., Jackson, P., and Baltimore, D. (1989). The mouse type IV c-abl gene product is a nuclear protein, and activation of transforming ability is associated with cytoplasmic localization. Cell *58*, 669-78.

Viswanatha, D., Chen, L., Liu, P., Slovak, M., Rankin, C., and Willman, C. (1996). Characterization and rapid diagnostic utility of a novel antibody detecting the cbf beta/smmhc fusion protein of inversion (16)/t(16;16) associated acute myeloid leukemia. Blood *88*, 664a.

Von Lindern, M., Fornerod, M., Van Baal, S., Jaeglé, M., de Wit, T., Buijs, A., and Grosveld, G. (1992a). The translocation (6;9), associated with a specific subtype of acute myeloid leukemia, results in the fusion of two genes, dek and can, and the expression of a chimeric, leukemia-specific dek-can mRNA. Mol Cell Biol *12*, 1687-1697.

Von Lindern, M., Van Baal, S., Wiegant, J., Raap, A., Hagemeijer, A., and Grosveld, G. (1992b). can, a putative oncogene associated with myeloid leukemogenesis, can be activated by fusion of its 3' half to different genes: characterization of the set gene. Mol Cell Biol *12*, 3346-3355.

Voncken, J. W., Kaartinen, V., Pattengale, P. K., Germeraad, W. T., Groffen, J., and Heisterkamp, N. (1995a). *BCR/ABL* P210 and P190 cause distinct leukemia in transgenic mice. Blood *86*, 4603-4611.

Voncken, J. W., Van Schaich, H., Kaartinen, V., Deemer, K., Coates, T., Landing, B., Pattengale, P., Dorseuil, O., Bokoch, G. M., Groffen, J., and Heisterkamp, N. (1995b). Increased respiratory burst in *bcr* null mutants. Cell *80*, 719-728.

Wang, J., Wang, M., and Liu, J. (1996a). Transformation properties of the eto gene, fusion partner in t(8;21) leukemias. Blood *88*, 556a.

Wang, Q., Stacy, T., Binder, M., Marín-Padilla, M., Sharpe, A. H., and Speck, N. A. (1996b). Disruption of the CBFα2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. Proc Natl Acad Sci USA *93*, 3444-3449.

Wang, Q., Stacy, T., Miller, J. D., Lewis, A. F., Gu, T.-L., Huang, X., Bushweller, J. H., Bories, J.-C., Alt, F. W., Ryan, G., Liu, P. P., Wynshaw-Boris, A., Binder, M., Marín-Padilla, M., Sharpe, A. H., and Speck, N. A. (1996c). The CBFβ subunit is essential for CBFα2 (AML1) function in vivo. Cell *87*, 697-708.

Wang, S., Wang, Q., Crute, B. E., Melnikova, I. N., Keller, S. R., and Speck, N. A. (1993). Cloning and characterization of subunits of the T-cell receptor and murine leukemia virus enhancer core-binding factor. Mol Cell Biol *13*, 3324-3339.

Wang, Z., Giorgio, M., Rivi, R., Cordon-Cardo, C., Gaboli, M., and Pandofi, P. (1996d). Growth and tumor suppressive properties of the PML gene of acute promyelocytic leukemia in PML-/- mice. Blood *88*, 476a.

Weis, K., Mattaj, I. W., and Lamond, A. I. (1995). Identification of hSRP1 alpha as a functional receptor for nuclear localization sequences. Science *268*, 1049-1053.

Weis, K., Rambaud, S., Lavau, C., Jansen, J., Carvalho, T., Carmo-Fonseca, M., Lamond, A., and Dejean, A. (1994). Retinoic acid regulates aberrant nuclear localization of PML-RAR alpha in acute promyelocytic leukemia cells. Cell *76*, 345-56.

Wen, W., Meinkoth, J. L., Tsien, R. Y., and Taylor, S. S. (1995). Identification of a signal for rapid export of proteins from the nucleus. Cell *82*, 463-473.

Wetzler, M., Talpaz, M., Van Etten, R. A., Hirsh-Ginsberg, C., Beran, M., and Kurzrock, R. (1993). Subcellular localization of Bcr, Abl, and Bcr-Abl proteins in normal and leukemic cells and correlation of expression with myeloid differentiation. J Clin Invest *92*, 1925-39.

Yergeau, D., Hetherington, C., Wang, Q., Zhang, P., Sharpe, A., Binder, M., Marín-Padilla, M., Tenen, D., Speck, N., and Zhang, D. (1996). AML1 fusion protein AML1/ETO directly blocks AML1 function during hematopoiesis. Blood *88*, 555a.

Zappavigna, V., L, F., MH, C., F, M., and ME, B. (1996). HMG1 interacts with HOX proteins and enhances their DNA binding and transcriptional activation. EMBO J *15*, 4981-91.

Zent, C. S., Mathieu, C., Claxton, D. F., Zhang, D. E., Tenen, D. G., Rowley, J. D., and Nucifora, G. (1996). The chimeric genes AML1/MDS1 and AML1/EAP inhibit AML1B activation at the CSF1R promoter, but only AML1/MDS1 has tumor-promoter properties. Proc. Natl. Acad. Sci. U.S.A. *93*, 1044-1048.

Ziemin-van der Poel, S., McCabe, N. R., Gill, H. J., Espinosa, R., III, Patel, Y., Harden, A., Rubinelli, P., Smith, S. D., LeBeau, M. M., and Rowley, J. D. (1991). Identification of a gene, MLL, that spans the breakpoint in 11q23 translocations associated with human leukemias [published erratum appears in Proc Natl Acad Sci USA 1992 May 1;89(9):4220]. Proc Natl Acad Sci USA *88*, 10735-10739.

Zwilling, S., Konig, H., and Wirth, T. (1995). High mobility group protein 2 functionally interacts with the POU domains of octamer transcription factors. EMBO J *14*, 1198-1208.

2 G_2 arrest and impaired nucleocytoplasmic transport in mouse embryos lacking the proto-oncogene *CAN/Nup214*

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G_2 arrest and impaired nucleocytoplasmic transport in mouse embryos lacking the proto-oncogene *CAN/Nup214*

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The vertebrate nucleopore complex (NPC) is a 125 MDa multiprotein assembly that mediates nucleocytoplasmic transport. One of its components, CAN/Nup214, is an FXFG repeat-containing protein known to be involved in myeloid leukemia in humans. We have devised a powerful genetic approach, using maternally derived protein in murine null embryos, to show that CAN/Nup214 is essential for NPC function *in vivo*. We demonstrate that *CAN–/–* mouse embryonic stem (ES) cells are not viable and that *CAN–/–* embryos die *in utero* between 4.0 and 4.5 days postcoitum, following the depletion of their CAN from maternal sources. In 3.5-day-old mutant embryos, cultured *in vitro*, progressive depletion of CAN leads to cell cycle arrest in G_2 phase, and eventually to blastocoel collapse, impaired NLS-mediated protein uptake and nuclear accumulation of polyadenylated RNA. Remarkably, these defective CAN-depleted embryos do not display any gross morphological abnormalities in their nuclear envelopes or NPCs. Our data suggest that CAN is critical to cell cycle progression and required for both nuclear protein import and mRNA export.

Keywords: CAN(Nup214)/cell cycle/gene targeting/nucleocytoplasmic trafficking/oncogenesis

Introduction

The human *CAN* gene was first identified as a target of chromosomal translocation (6;9)(p23;q34) associated with a subtype of acute myeloid leukemia (von Lindern *et al.*, 1992). CAN, also called Nup214, is a nuclear pore complex (NPC) protein: it associates with the NPC and its primary sequence contains multiple copies of NPC protein-specific peptide repeat motifs (Kraemer *et al.*, 1994; Fornerod *et al.*, 1995). Some 100 different proteins (nucleoporins) make up the NPC, a 125 MDa supramolecular structure that crosses the nuclear envelope and mediates bidirectional transport of macromolecules between the cytoplasm and the nucleus (reviewed by Rout and Wente, 1994; see also Panté and Aebi, 1994; Davis, 1995; Doye and Hurt, 1995; Görlich and Mattaj, 1996; Hurt, 1996).

Selective protein import into the nucleus is a two-step process. First, a heterodimeric complex composed of the nuclear localization signal (NLS) receptor and p97 binds to a karyophilic substrate in the cytosol. The NLS receptor of the heterodimer recognizes the substrate's NLS (Adam and Gerace, 1991; Adam and Adam, 1994; Görlich *et al.*, 1994), and p97 may mediate binding of the import complex to FXFG repeat-containing nucleoporins associated with the NPC (Chi *et al.*, 1995; Görlich *et al.*, 1995; Moroianu *et al.*, 1995a; Radu *et al.*, 1995a). Second, the entire complex moves through the central channel of the NPC. This process is energy-dependent and mediated by the small GTPase Ran (Melchior *et al.*, 1993; Moore and Blobel, 1993). The import complex components are then released into the nucleoplasm, except for p97, which is retained at the nuclear side of the NPC (Görlich *et al.*, 1995; Moroianu *et al.*, 1995a). Export of proteins and ribonucleoproteins (RNPs) from the nucleus is also an active, energy-dependent and factor-mediated process, and short protein sequences that act as nuclear export signals (NESs) have recently been identified in some of these factors (Bogerd *et al.*, 1995; Fischer *et al.*, 1995; Fritz *et al.*, 1995; Gerace, 1995; Stutz *et al.*, 1995; Wen *et al.*, 1995; Görlich and Mattaj, 1996).

Over the past few years, a number of NPC proteins have been identified in yeast and biochemical and genetic approaches have shed some light on their roles in nucleocytoplasmic transport (Davis, 1995; Doye and Hurt, 1995; Rexach and Blobel, 1995). Yet, the lack of both accurate protein import assays and experiments that localize the proteins within the NPC, together with pleiotropic defects in some nucleoporin mutants, have hampered precise interpretation of functional studies. In higher eukaryotes, most nucleoporins identified to date have been sublocalized to specific NPC regions (Panté and Aebi, 1994), but their *in vivo* function(s) remain to be explored by genetic tools.

Results

CAN is an essential nucleoporin

As a first step towards genetic dissection of the NPC we disrupted the murine *CAN* gene by using gene targeting and ES cell technology (Figure 1A). Heterozygous mutant mice were interbred and their offspring genotyped by Southern blot analysis (Figure 1B). None of the 233 pups screened was homozygous for the disrupted *CAN* allele, demonstrating that CAN is essential for embryonic development. To determine the time at which the CAN gene disruption is lethal, 3.5-, 6.5-, 7.5- and 9.5-day-old embryos were isolated and their genotypes were determined by polymerase chain reaction (PCR) amplification of DNA fragments diagnostic for the wild-type and the disrupted *CAN* allele (data not shown). No *CAN–/–* embryos were found at days 6.5, 7.5 and 9.5 of gestation; however, at 3.5 days postcoitum, *CAN–/–* embryos were detected at a normal Mendelian frequency of 25%. These homozygous mutant blastocysts were morphologically indistinguishable from their wild-type and heterozygous counterparts.

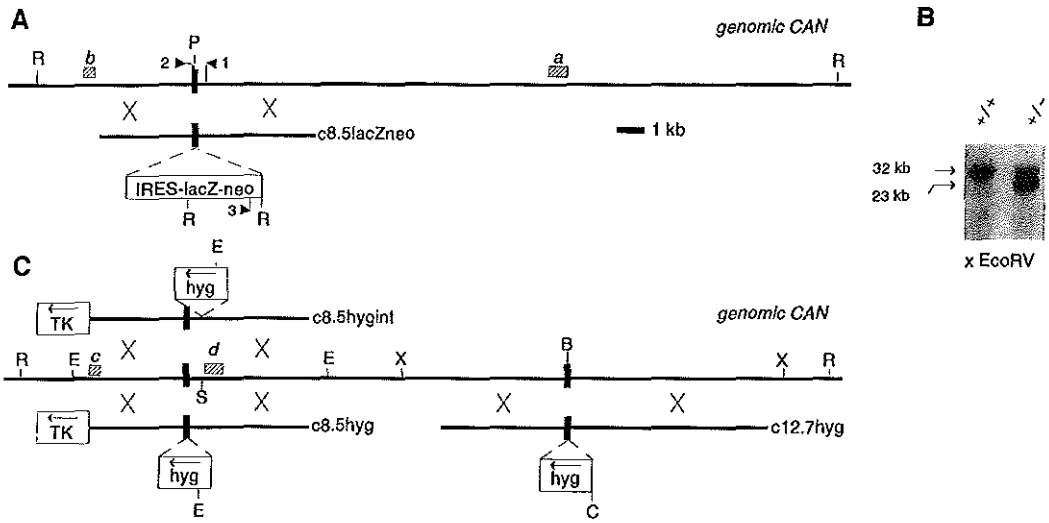

Fig. 1. Targeted disruptions of the mouse *CAN* gene in ES cells. (A) Schematic diagram showing a 32 kb *Eco*RV fragment of the mouse *CAN* locus and primary targeting vector c8.5lacZneo. The solid box indicates the mouse exon that corresponds to codons 278–429 of the human *CAN* gene. Shaded boxes mark probes used for Southern blot screening of homologous recombinants and arrowheads indicate the positions of the three primers used to PCR genotype the embryos. (B) Southern blot analysis of wild-type (+/+) E14 ES cells and targeted ES clone 65 (+/–). *Eco*RV-digested DNA hybridized to probe *a*. Arrows indicate the positions of the wild-type (32 kb) and lacZneo-disrupted alleles (23 kb). (C) Schema of the wild-type *CAN* allele of heterozygous mutant ES cells and the three targeting vectors used in the second rounds of homologous recombination. Restriction sites and probes (*c* and *d*, shaded boxes) used in Southern blot analysis are indicated. Abbreviations: TK, HSV-tk gene; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; P, *Pvu*II; R, *Eco*RV; S, *Stu*I; X, *Xba*I.

To evaluate the role of CAN further, we wanted to inactivate the remaining functional *CAN* allele in heterozygous ES cells. First, *CAN+/–* ES cells were selected in high concentrations of G418 (Mortensen *et al.*, 1992), but none of the 34 resistant clones showed duplication of the mutant *CAN* allele. Second, *CAN+/–* ES cells were subjected to a second round of homologous recombination by using vector c8.5hyg (Figure 1C). Twelve targeted ES clones were obtained; strikingly, all homologous recombination events occurred at the previously disrupted allele. A second double-targeting vector, c12.7hyg (Figure 1C), yielded 105 targeted clones but, again, all recombination events occurred at the previously disrupted *CAN* allele. The above results strongly suggest that inactivation of both *CAN* alleles is incompatible with ES cell survival.

To exclude the possibility that the wild-type allele in the *CAN+/–* ES clones was refractory to homologous recombination, we constructed and tested a third double-targeting vector, c8.5hygint (Figure 1C). This vector was designed to insert a selectable marker in the *CAN* gene without disrupting its coding sequence. Nine of 23 double-targeted *CAN+/–* clones showed recombination at the wild-type allele, thereby indicating that this allele is accessible to homologous recombination, and supporting our conclusion that CAN is essential for cell survival.

How can homozygous null embryos survive during the early steps of development, yet ES cells that lack the CAN protein die? To test whether maternally derived CAN (Kidder, 1992) could account for the normal development of homozygous mutants during very early embryogenesis, we immunostained fertilized eggs (0.5 day postcoitum; $n=35$), 2-cell embryos (1.5 days postcoitum; $n=24$), 8/16-cell embryos (2.5 days postcoitum; $n=39$), blastocysts (3.5 days postcoitum; $n=15$) and hatched blastocysts (4.0 and 4.5 days postcoitum; $n=15$ and $n=39$, respectively) with affinity-purified anti-CAN antibodies (αCNN76-2). We found that the intensity of staining of 0.5-, 1.5- and 2.5-day-old mutant embryos was indistinguishable from that in wild-type and heterozygous embryos (Figure 2A and B). In contrast, cells of *CAN–/–* blastocysts stained very weakly for CAN (Figure 2C and D) and *CAN–/–* day 4.0 hatched blastocysts did not stain at all (Figure 2E and F). At 4.5 days no CAN-negative hatched blastocysts were observed. These results indicate that CAN levels appear normal in mutant embryos up to 2.5 days postcoitum; however, over the next 24 h CAN levels drop and the embryos die between day 4.0 and 4.5 because of the ongoing CAN depletion.

Growth arrest and degradation of cultured CAN–/– embryos

To understand the degeneration of *CAN–/–* embryos better, we isolated blastocysts from matings between heterozygous mutant mice, cultured them, and examined them microscopically. Initially, wild-type, heterozygous and homozygous mutant blastocysts, genotyped by PCR, were morphologically indistinguishable (Figure 3A and B). However, after 18–24 h, the blastocoel of homozygous mutant embryos ($n=22$) gradually became smaller until it had completely collapsed 2–3 h later (Figure 3B–D). Trypan blue staining of these collapsed embryos revealed no signs of cell death. In contrast, 91% of wild-type ($n=23$) and 96% of heterozygous embryos ($n=44$) hatched

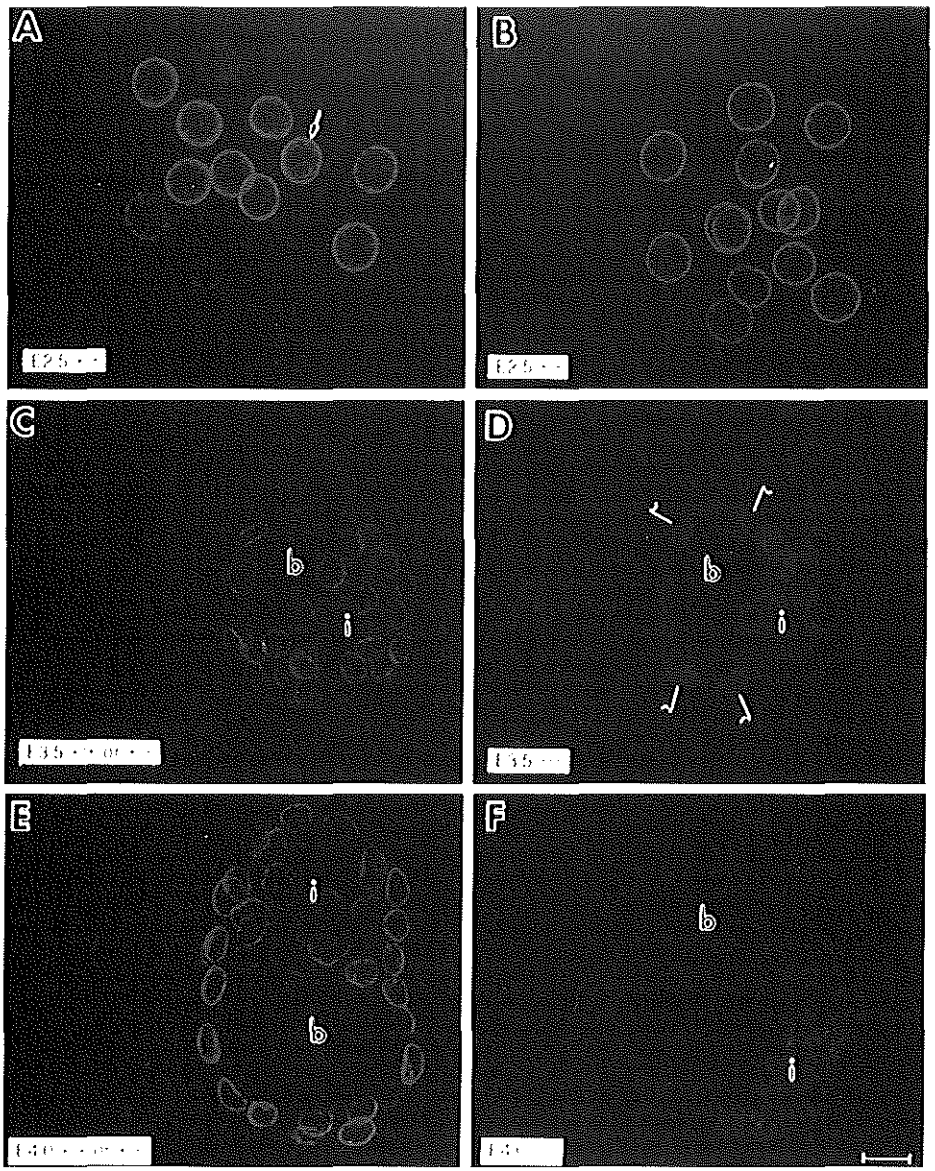

Fig. 2. Indirect immunofluorescence detection of CAN in pre-implantation embryos from *CAN+/–* interbreedings. Representative confocal images of 2.5- to 4-day-old embryos stained with affinity-purified anti-CAN antibodies (αCNN76-2). (**A**) Cells of a heterozygous mutant embryo at day 2.5 showing CAN-specific staining of the nuclear membrane, indicated by the arrow. (**B**) Nuclear envelopes of embryonic cells from a 2.5-day-old homozygous mutant embryo stained with similar intensity as normal embryonic cells. (**C** and **E**) In wild-type or heterozygous embryos (day 3.5 and 4), CAN is localized to the nuclear membrane of inner cell mass cells (labeled i) and of the trophectoderm cells that shape the blastocoel (labeled b). (**D**) In mutant 3.5-day-old embryos, CAN-specific staining is barely detectable; arrowheads point to cells with weak nuclear envelope staining. (**F**) A hatched *CAN–/–* blastocyst, 4 days postcoitum, does not stain for CAN. The scale bar in (F) represents 14 μm.

normally from the zona pellucida and attached to the culture dish during the second day of culture.

Mutant and control blastocysts contained similar numbers of cells at the start of culture [38±10 (n=3) versus 37±5 (n=26)] cells, respectively). In contrast, at the onset of blastocoel contraction, *CAN–/–* embryos contained considerably fewer cells than did controls [45±7 (n=12) versus 76±4 (n=11) cells, respectively], indicating that very few cells of the mutant blastocyst cells had divided in culture.

We then examined whether the cells of *CAN–/–* blastocysts were arrested at a particular stage of the cell

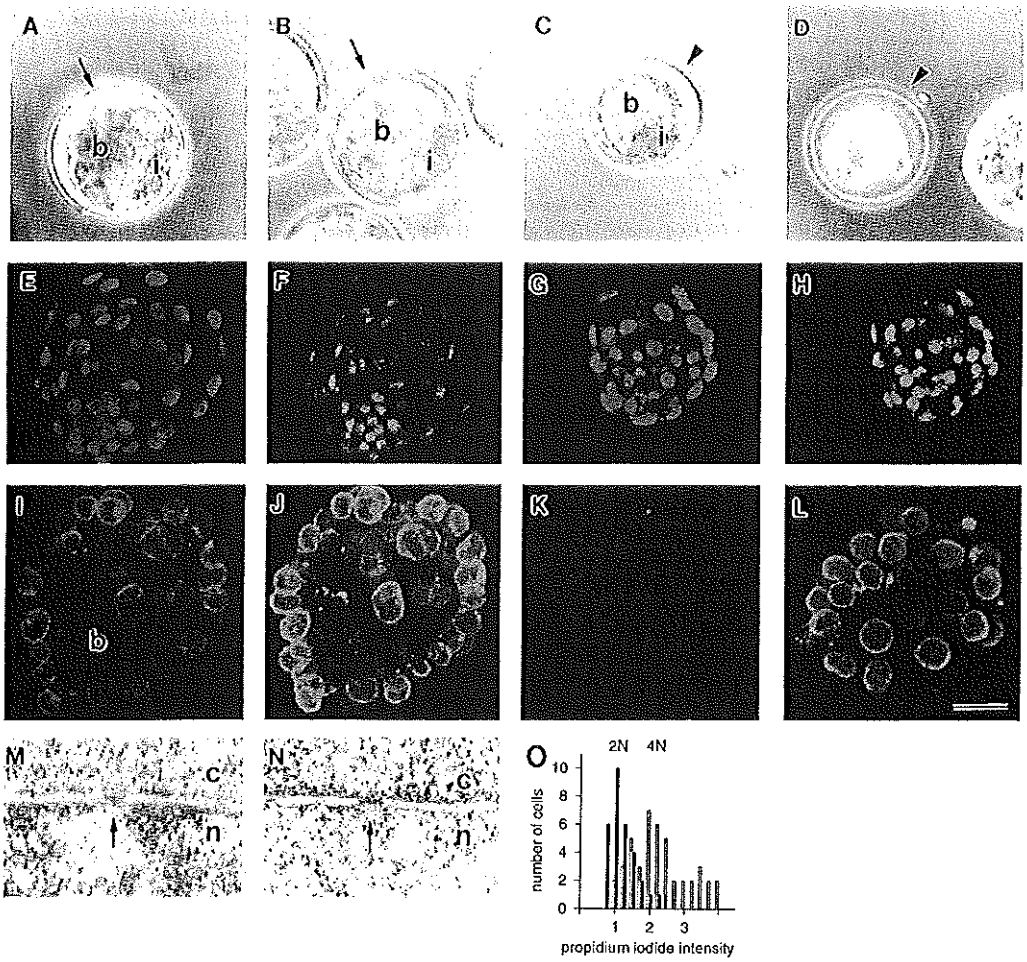

Fig. 3. Growth and morphology of CAN-depleted blastocysts cultured *in vitro*. (A–D) Phase contrast images of representative 3.5-day-old embryos cultured *in vitro*. (A) Wild-type embryo cultured for 18 h; the morphology of the embryo remained unchanged over the next 5 h. (B–D) A *CAN–/–* embryo at various stages of *in vitro* culture: (B) at t =18 h (~1 h before the onset of its blastocoel collapse); (C) 2 h later; (D) 4 h later. Arrows indicate trophectoderm cells; b, blastocoel; i, inner cell mass. Arrowheads point to the zona pellucida. (E–H) DNA replication and DNA content of blastocysts cultured *in vitro* for 20 h in the presence of BrdU. (E and F) Confocal images of a control blastocyst stained for BrdU (E) and propidium iodide (F). (G and H) A representative *CAN–/–* embryo at the onset of its blastocoel collapse (at ~20 h of *in vitro* culture) stained for BrdU (G) and propidium iodide (H). Note that almost all mutant nuclei stain strongly for propidium iodide. (E and G) and (F and H) were scanned and photographed under identical conditions. (I–L) Nuclear envelope morphology and NPC distribution as viewed by immunofluorescence microscopy. (I and J) Confocal images of a control blastocyst (cultured for 24 h) stained with (I) αCNN76-2 and (J) mAb414 (10 μg/ml). (K and L) A representative CAN-depleted embryo 2 h after onset of its blastocoel remission (t = 26 h of *in vitro* culture) stained with (K) αCNN76-2 and (L) mAb414. (M and N) Electron micrographs of the nuclear envelopes of control and degenerating embryos. Detail of (M) a normal and (N) a collapsed blastocyst (~2 h after onset of blastocoel contraction). n, nucleus; c, cytoplasm; arrows indicate the nuclear pores. The bar in (L) represents the length of 30 μm in (A–D), 36 μm in (E–H), 18 μm in (INL), and 155 nm in (M) and (N). (O) Histogram of the DNA content of control (black) and mutant embryonic cells (gray) after 20 h of culture derived from cytometric analysis. Propidium iodide fluorescence is indicated in arbitrary units. 2N, unreplicated DNA; 4N fully replicated DNA.

cycle. For this study, 3.5-day-old embryos from *CAN+/–* intercrosses were isolated and cultured in the presence of bromodeoxyuridine (BrdU) until the *CAN–/–* embryos started to collapse (18–24 h). The BrdU incorporated into the DNA during S phase was then assessed by using indirect immunofluorescence with an anti-BrdU antibody. The embryos were also stained for DNA with a fluorescent marker, propidium iodide, to quantitate the total DNA content of their cells by image cytometric measurements. BrdU did not affect growth and development of mouse embryos *in vitro*. All of the nuclei of both control embryos (n=7; Figure 3E) and *CAN–/–* embryos (n=5 embryos; Figure 3G) stained for BrdU, which demonstrated the ability of *CAN–/–* cells to enter and progress through S phase. In contrast to embryonic nuclei of controls (Figure 3F), the vast majority of nuclei of the mutant embryos

stained strongly for propidium iodide (Figure 3H). Cytometric quantitation of propidium iodide stained nuclei revealed that 75–85% of cells from *CAN–/–* embryos contained a 4N equivalent of DNA. On the other hand, only 10–15% of cells in *CAN+/–* and *CAN+/+* embryos showed a 4N DNA content: the majority of cells from the control embryos undergo mitosis during the 18–24 h culture period and therefore show a DNA content of ~2N (Figure 3O). These results indicate that *CAN–/–* cells seem to progress through S phase and become arrested in G_2. As a control, we arrested cells of wild-type embryos (n=16) in G_2 by treating them with Hoechst 33342 (0.15 μg/ml, n=10; Tobey *et al.*, 1990), and found that the G_2 arrest does not induce blastocyst degeneration.

To test whether *in vitro* degeneration of murine *CAN–/–* embryos is accompanied by alterations in the morphology of the nuclear envelope or NPCs, as has been shown in yeast (Doye *et al.*, 1994; Wente and Blobel, 1994; Gorsch *et al.*, 1995; Li *et al.*, 1995), embryos from *CAN+/–* intercrosses were stained with a monoclonal antibody (mAb414; Davis and Blobel, 1986), to O-glycosylated nucleoporins. Indirect immunofluorescence showed a punctate nuclear rim staining in all cells of the *CAN–/–* embryos for at least 6 h after the onset of blastocoel remission (Figure 3L). This staining pattern was indistinguishable from that seen in *CAN+/+* and *CAN+/–* embryos (Figure 3J). Moreover, there was no evidence of NPC clustering. We also did not observe fractionated nuclei, indicating that there were no apoptotic cells in these collapsed embryos.

Ultrastructural examination of thin-sectioned *CAN–/–* embryos (0.5 and 2 h after initiation of blastocoel remission) revealed no herniations, invaginations (Wente and Blobel, 1993, 1994), or other abnormalities of either the nuclear envelope or the NPCs (Figure 3M and N). Together, these data show that CAN depletion does not induce major structural changes in the nuclear envelope or the NPCs, and that the death of *CAN–/–* cells is likely caused by functional rather than structural deficiencies.

Impaired NLS-mediated nuclear protein import

We examined whether CAN depletion affects nuclear protein import in embryonic cells by assaying the *in vitro* import of fluorescent allophycocyanin protein (APC) chemically coupled to the SV40 large T NLS peptide (APC–NLS; Adam *et al.*, 1990). We assessed APC–NLS accumulation at the onset of blastocoel contraction in the nuclei of digitonin-permeabilized cells from *CAN–/–* and control embryos after 5 and 10 min incubations at 25°C. As shown in Figure 4A–D, the nuclei of both control and CAN-depleted embryos at the onset of blastocoel contraction accumulated increasing amounts of APC–NLS during the assay period; however, the rate of import was always significantly lower for the mutants (P<0.05). The average nuclear concentration of APC–NLS after 5 min incubations was 45–60% lower for the *CAN–/–* embryos (n=8; Figure 4B) than for the control embryos (n=8; Figure 4A) in the same import reaction. Import of APC–NLS was selective because APC without an NLS did not accumulate in the embryonic nuclei (n=4 embryos; Figure 4I). We also analyzed nuclear import at two earlier stages of *in vitro* culture when *CAN–/–* embryos appeared morphologically indistinguishable from controls. Nuclear uptake of APC–NLS in mutant embryos that had been cultured for 12 h (n=4) was similar to that in control embryos cultured for the same amount of time (data not shown). In contrast, after about 20 h in culture, four *CAN–/–* embryos (compare Figure 4G and H) showed impaired NLS-mediated protein import (24–33% less APC–NLS imported compared with control embryos), whereas seven other *CAN–/–* embryos still displayed similar import as controls (compare Figure 4E and F). These results show that protein import becomes defective shortly before the onset of blastocoel contraction.

Recent results suggest that p97 may mediate docking of cytosolic import complexes to repeat-containing nucleoporins associated with the NPC (Radu *et al.*, 1995b). To test for abnormalities in p97 binding to CAN-depleted NPCs, we immunostained embryos derived from *CAN+/–* interbreedings with a monoclonal antibody to p97 (mAb3E9; Chi *et al.*, 1995). We observed a strong punctate nuclear rim staining in the cells of the controls (Figure 4J) and of the *CAN–/–* embryos at different stages of blastocoel contraction (1, 3 and 6 h after onset; Figure 4K). It demonstrates that CAN-depleted NPCs can bind p97, suggesting that the p97-mediated docking of nuclear proteins to the NPC is not solely dependent on CAN.

We also examined the *in vivo* localization of the nuclear protein DEK by using immunofluorescence with anti-DEK antibodies (Fornerod *et al.*, 1995). DEK localized to the nucleus of *CAN–/–* embryos stained 1 and 3 h after the onset of the blastocoel collapse (data not shown). In addition, we analyzed the subcellular distribution of the heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) in mutant embryos at the onset of blastocoel contraction. hnRNP A1 plays an important role in the biogenesis of mRNA. It shuttles continuously between the nucleus and the cytosol, although it localizes predominantly to the nucleus (Piñol-Roma and Dreyfuss, 1992). Nuclear uptake of hnRNP A1 is mediated by a 38 amino acid sequence, termed M9, that is distinct from classical NLS sequences, and also functions as an NES to mRNA export from the nucleus (Michael *et al.*, 1995; Siomi and Dreyfuss, 1995). Using a monoclonal antibody against hnRNP A1 (9H10) to immunostain *CAN–/–* (n=5) and control embryos (n=7) at the onset of blastocoel contraction, we found that hnRNP A1 localized properly and did not accumulate in the cytoplasm of *CAN–/–* embryos (Figure 4L and M).

Nuclear accumulation of poly(A)$^+$ RNA

We next examined embryonic cells for nuclear export of mRNA. Subcellular localization of poly(A)$^+$ RNA in mutant embryos was studied at various time points before and after onset of blastocoel contraction by *in situ* hybridization with an oligo(dT)$_{50}$ probe directly coupled to FITC (Figure 5A, C, E and G). For reference in each of the hybridized embryos the position of the nuclei is shown by double staining with propidium iodide (Figure 5B, D, F and H). In the cells of control embryos (n=12), poly(A)$^+$ RNA was diffusely distributed in the nucleoplasm and cytoplasm (Figure 5A). By contrast, in *CAN–/–* embryos (n=14) that were at the onset of blastocoel contraction or 1 to 4 h thereafter (Figure 5C), we found strong nuclear staining, demonstrating nuclear accumulation of poly(A)$^+$ RNA. As control on the specificity of our mRNA detection method, embryos pretreated with RNase were stained.

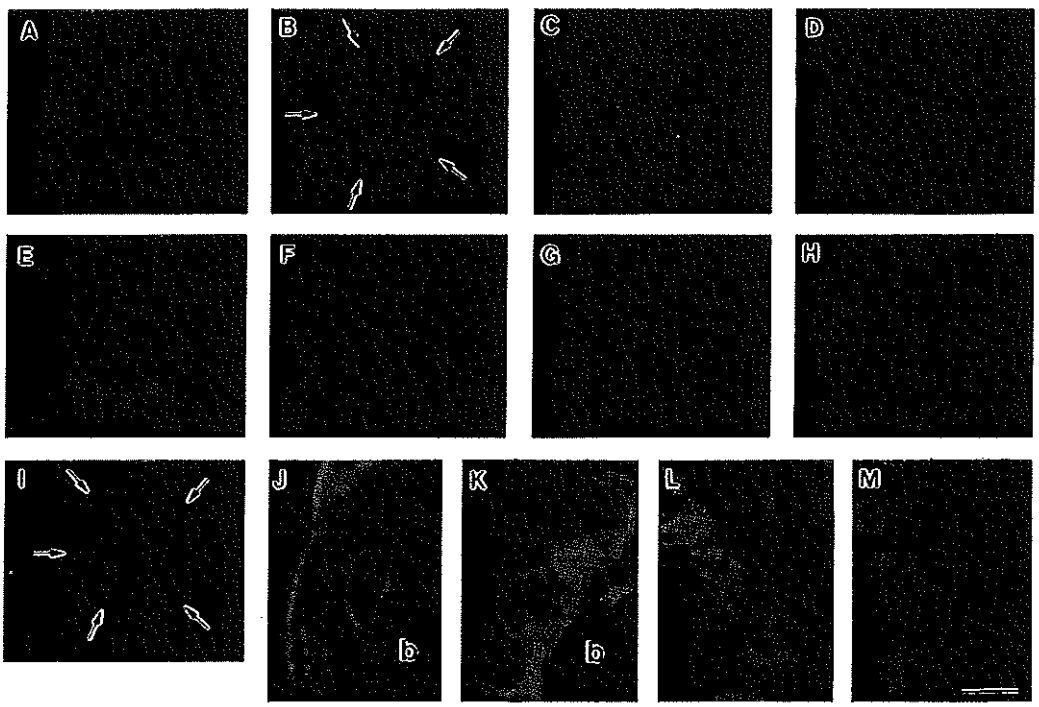

Fig. 4. Nuclear import of karyophilic substrates and subcellular distributions of p97 and hnRNP A1. (A–H) Representative nuclear protein import assays on cultured blastocysts. Import of APC–NLS into the nuclei of control (A and C) and *CAN–/–* embryos at the onset of blastocoel collapse (after 20.5 h of culture) (B and D), after 5 min (A and B), and after 10 min (C and D) incubations in transport mixture. All four embryos were from the same import experiment. The average nuclear concentration of APC–NLS was 45% lower in (B) than in (A), and 52% lower in (D) than in (C). Import of APC–NLS into the nuclei of control (E and G) and *CAN–/–* embryos (F and H) cultured for 20 h (but lacking any signs of blastocoel collapse) incubated for 5 min in transport mixture. The average nuclear concentration of APC–NLS was similar in (E) and (F), but was 30% lower in (H) than in (G). (I) Representative embryo incubated with APC that lacks an NLS. All embryos were examined with a conventional immunofluorescence microscope and photographed under identical conditions. Not all of the fluorescent nuclei were in the same focal plane. Nuclei in the center of embryos (E), (F) and (H) that were not in focus appeared as background fluorescence. (J–K) Confocal microscopy of embryos immunostained with an antibody to p97 (mAb3E9; 2 μg/ml). p97 localized to the nuclear membrane and the cytoplasm (weak staining) of control (J) and *CAN–/–* embryonic cells (K; 1 h after onset of blastocoel remission). (L–M) Confocal images of embryos immunostained with a monoclonal antibody to hnRNP A1 (mAb9H10; 1 in 500 dilution). hnRNP A1 predominantly localizes to the nuclei of both control (L) and *CAN–/–* (M) embryos at the onset of blastocoel contraction. Unstained areas represent cytoplasmic compartments of embryonic cells. Abbreviation: b, blastocoel. The outline of embryos (B) and (I) marked by arrows. The scale bar in (M) represents 18 μm in (A–I), and 8 μm in (J–M).

Typically, such embryos showed little or no cytoplasmic or nuclear staining (Figure 5G and H).

We also assayed the mRNA export status at two earlier stages of culture, when mutants and controls were still microscopically indistinguishable. *CAN–/–* embryos (n=4 embryos) grown *in vitro* for 12 h, had normal poly(A)$^+$ RNA distribution patterns (data not shown). Yet, after 20 h of culture, two out of four homozygous mutant embryos had begun to accumulate poly(A)$^+$ RNA (Figure 5E and F). This result shows that the block in RNA export precedes the onset of blastocoel contraction.

Discussion

We have demonstrated that expression of CAN/Nup214, an FXFG repeat-containing component of the NPC, is critical to NLS-mediated nuclear protein import and poly(A)$^+$ RNA export. Radu *et al.* (1995b) have recently shown that repeat-containing nucleoporins, immobilized on nitrocellulose, can bind a model substrate in the presence of the NLS receptor and p97. This led to the hypothesis that bidirectional transport across the vertebrate NPC may require transport substrates to dock and undock at an array of repeat-containing nucleoporins across the NPC (Moroianu *et al.*, 1995a,b; Radu *et al.*, 1995a). Our finding that NLS-mediated nuclear protein import is significantly reduced in CAN-depleted embryos is consistent with, and provides the first *in vivo* support for this hypothesis. The residual nuclear protein uptake in CAN-depleted embryonic nuclei may reflect some functional compensation by other repeat-containing proteins that are associated with the NPC. Alternatively, it may result from trace amounts of CAN in the NPCs.

Only very few cells of the *CAN–/–* blastocysts divide during the 18–24 h of *in vitro* culture. BrdU labeling experiments and DNA content analysis suggest that these cells are arrested in G_2 phase of their cell cycle. Interestingly, cells expressing *srp1-31*, a temperature-sensitive mutant of the *Saccharomyces cerevisiae* NLS receptor, are also defective in nuclear protein import and arrest in

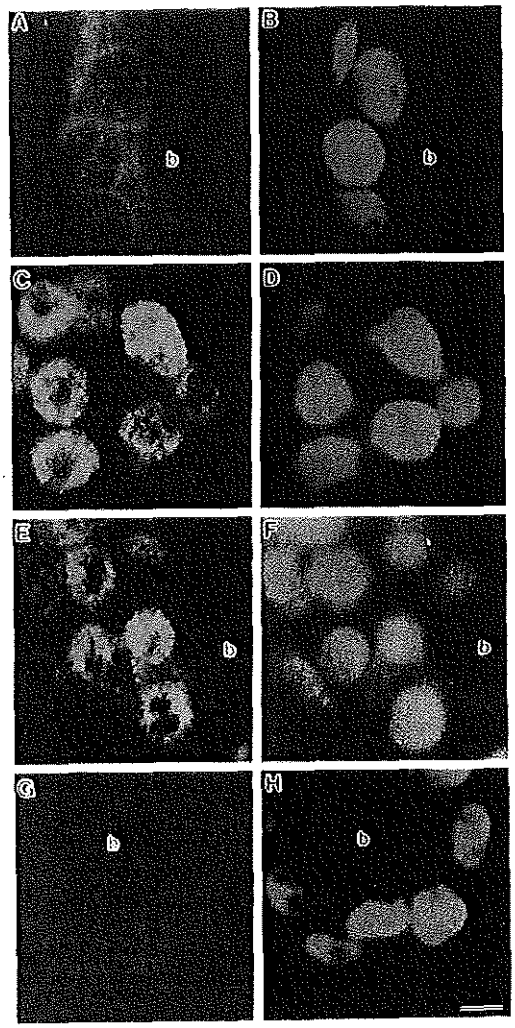

Fig. 5. CAN depletion causes poly(A)$^+$ RNA accumulation in embryonic nuclei. (**A, C, E** and **G**) Subcellular localization of polyadenylated RNA in embryonic cells analyzed by *in situ* hybridization with an FITC-labeled oligo(dT)$_{50}$ probe. (**B, D, F** and **H**) Coincident DNA staining with propidium iodide. (A and B) Wild-type embryo (in culture for 24 h); (C and D) embryo homozygous for the disrupted CAN allele, ~2 h after onset of its blastocoel remission (in culture for 24 h); (E and F) *CAN–/–* embryo showing no signs of degeneration after 20 h in culture; (G and H) wild-type embryo pretreated with RNase. Cells from this embryo displayed little nuclear or cytoplasmic staining. Blastocoels are denoted by b. The bar in (H) represents 7 μm.

the cell cycle during the G_2/M phase when cultured under non-permissive conditions (Loeb *et al.*, 1995). The G_2/M arrest in *srp1-31* cells presumably results from a block in import of nuclear protein(s) that mediate a cell cycle-regulated wave of proteolysis that is known to trigger progression through M phase (Amon *et al.*, 1994; Loeb *et al.*, 1995). It is conceivable that the G_2 arrest in the *CAN–/–* embryos is also induced by sensitivity of the cell cycle machinery to reduced nuclear protein import capacity.

CAN–/– embryos not only have impaired NLS-mediated protein import, but their nuclei also accumulate poly(A)$^+$ RNA. These two defects in nucleocytoplasmic transport coincided as CAN was depleted, and may reflect a dual role in protein import and mRNA export, as has been reported for the yeast nucleoporin *NUP49* (Doye *et al.*, 1994). Some temperature-sensitive *NUP49* mutants are primarily defective in nuclear protein import, while in others the initial transport defect occurs at the level of mRNA export. A dual role for CAN in both import and export could be achieved via its FXFG repeats. The FXFG repeats may serve *in vivo* as a docking site for nuclear import of proteins carrying a classical NLS, and they could also have some function in RNA export. This is supported by the remarkable homology between the FXFG repeats of CAN and those of the novel human nucleoporin-like protein RIP/RAB (Bogerd *et al.*, 1995; Fritz *et al.*, 1995). The FXFG repeat-containing domain of RIP/RAB is sufficient for binding to the NES of HIV-1 Rev in a yeast two-hybrid assay, which suggests that the repeats may function as an NES receptor for selective export of viral mRNAs from the nucleus (Gerace, 1995). By the same token, the FXFG motif of CAN may function as an NES receptor in RNA export.

The *srp1-31* mutation blocks NLS-mediated import without causing poly(A)$^+$ RNA accumulation in the nucleus (Loeb *et al.*, 1995). From this observation, we infer that a basic defect in protein uptake does not hinder mRNA export. It supports our contention that the block in mRNA export may be caused by a second function of CAN that is independent from its role in NLS-mediated protein uptake. Like a number of other nucleoporins (Siniossoglou *et al.*, 1996), CAN could be part of one or more subcomplexes within the NPC, and its depletion may simultaneously lead to dissociation of one or more additional nucleoporins from the NPC, for example one that functions directly in mRNA export or protein import.

Just as proteins are selectively imported into the nucleus, RNA export must be carefully controlled. RNP particles move from the site of transcription and assembly to the NPC, where they are translocated to the cytoplasm (Izaurralde and Mattaj, 1995). Nuclear accumulation of poly(A)$^+$ RNA could, therefore, be an indirect consequence of decreased nuclear import of proteins critical to mRNA export that do not employ the classical NLS-mediated pathway for their transport into the nucleus. A prime candidate for such a protein would be hnRNP A1 (Michael *et al.*, 1995). We found no mislocalization of this protein in the cytoplasm of *CAN–/–* cells at a stage when poly(A)$^+$ RNA was retained in the nucleus. This result suggests that the export defect cannot be attributed to the consequences of impaired hnRNP A1 import. It remains to be seen whether this observation holds true for other proteins involved in the poly(A)$^+$ RNA export pathway. The apparently normal nuclear uptake of hnRNP A1 by cells lacking CAN suggests that the M9-mediated nuclear import pathway is independent of CAN.

To conclude, CAN/Nup214 is the first nucleoporin gene to be disrupted in higher eukaryotes to study its function in nucleocytoplasmic transport *in vivo*. We have shown that the presence of maternal gene products in early

embryogenesis permits the use of gene knockout strategies to study the function of genes that are essential for cell survival.

Materials and methods

Targeted disruption of the CAN locus in ES cells

Vector c8.5lacZneo contained an 8.5 kb *Bam*HI–*Sal*I CAN 129Sv/E genomic fragment with an IRES-lacZneo selection cassette (Mountford and Smith, 1995) inserted into a unique *Pvu*II site that corresponds to codon 297 of the human *CAN* gene. Vector c8.5hyg consisted of the same 8.5 kb genomic *CAN* fragment. A hyg^r marker was positioned in its *Pvu*II site and a herpes simplex virus thymidine kinase (HSV-tk) gene cassette was included for negative selection (van Deursen and Wieringa, 1992). Vector c8.5hygint is identical to c8.5hyg except that the hyg^r marker was inserted into an intronic *Sta*I site. Vector c12.7hyg consisted of a 12.7 kb *Hin*dIII *CAN* fragment (from a 129Sv/E genomic library) with a hyg^r marker inserted into a unique *Bam*HI site that corresponds to codon 804 of the human *CAN* gene. These targeting vectors were linearized and electroporated into wild-type or *CAN*+/– E14 mouse ES cells, drug-resistant ES lines isolated, and targeted clones identified for correct replacement events by Southern blot analysis using external probes on *Eco*RV- (c8.5lacZneo; probe a), *Eco*RI- (c8.5hyg and c8.5 hygint; probe c) or *Eco*RV/*Cla*I-cut genomic DNA (c12.7hyg; probe d) as previously described (van Deursen and Wieringa, 1992). Mutant mice were generated as previously described (van Deursen *et al.*, 1993).

Isolation, culture and genotype analysis of mouse embryos

Heterozygous mutant mice, kept on a 6 a.m. to 6 p.m. light–dark schedule, were mated for timed pregnancies. The vaginal plug was scored at 12 p.m. which we designated 0.5 day of development (0.5 day postcoitum). At various times of development, we collected embryos from the uterine horns of plugged *CAN*+/– female mice as described by Hogan *et al.* (1994). Blastocysts, were cultured *in vitro* as described by Hsu (1979).

Embryos were genotyped by PCR amplification. Individual embryos were transferred to Eppendorf tubes containing 1 µl sterile water, to which 3 µl lysis buffer [0.05% SDS (w/v) and 0.035 N NaOH] was added. The samples were boiled for 3 min, and 1.5 µl of this mixture was used for PCR. Embryos at day 6.5, 7.5 and 9.5 were washed extensively in PBS before being transferred to 50 µl of PCR protK buffer [60 mM Tris–HCl, pH 9.0, 15 mM $[NH_4]_2SO_4$, 2 mM $MgCl_2$, 0.5% Tween-20 (v/v) and 250 µg/ml proteinase K]. After incubation for 5 h at 56°C, the proteinase K was inactivated by boiling for 10 min, and 3 µl from each sample was used for PCR. PCR cocktails containing primers (see below) that were diagnostic for the wild-type and lacZneo-disrupted allele were added to the embryo lysates [final concentrations, 60 mM Tris–HCl, pH 9.0, 15 mM $[NH_4]_2SO_4$, 2 mM $MgCl_2$, 200 µM dNTPs, 250 ng of each primer, 180 ng of TaqStart antibody (Clonetech), and 0.8 unit Taq DNA polymerase (Perkin-Elmer Cetus)]. Samples were amplified for 30–35 cycles (94°C for 1 min, 55°C for 2 min, 72°C for 2 min), and PCR products were resolved by electrophoresis through 1.5% agarose gels. The identity of the PCR fragments was confirmed by Southern blot analysis, using an internal CAN oligonucleotide (5′-CACTCCTCGATGTAGCTGAGG-3′) as a probe.

Sequences specific for the wild-type allele were amplified by using, anti-sense (5′-TGGAATCACTCACTGTGGTTG-3′; Figure 1A, primer 1) and sense (5′-GTGAACTTCATGGAGCCCTG-3′; Figure 1A, primer 2) primers that flank the *Pvu*II site within the targeted *CAN* exon (Figure 1A). The targeted allele sequences were amplified by using the same anti-sense *CAN* primer in combination with a sense strand primer for the neo^r gene (5′-TCGTGCTTTACGGTATCGC-3′; Figure 1A, primer 3). These PCR fragments measure 430 bp (wild-type) and 860 bp (lacZ-disrupted), respectively.

Antibody purification and indirect immunofluorescence

Polyclonal rabbit anti-CAN antibodies directed against the N-terminal part of human CAN (αCNN) were affinity-purified by adsorption for 18 h at 4°C to a bacterially produced polypeptide representing amino acids 363–804 of human CAN (Fornerod *et al.*, 1995) bound to Immobulon PVDF membrane (Millipore Corp., Bedford, MA). The membrane was washed five times with PBS containing 0.05% Tween-20 (v/v), and then washed once with PBS alone. Bound antibodies were eluted for 3 min at 0°C in a buffer containing 100 mM glycine at pH 2.5 and 0.05% (w/v) bovine serum albumin. They were then immediately neutralized with 0.05 volumes of 1 M $NaPO_4$ at pH 7.5. The purified antibodies are designated αCNN76-2.

Indirect immunofluorescence was as previously described (Fornerod *et al.*, 1995). Antibody dilutions were 1 in 16 for αCNN76-2, 10 µg/ml for protein A-purified mAb414 (BAbCO), 2 µg/ml for mAb3E9 (Chi *et al.*, 1995), 1 in 1000 for αDEK (Fornerod *et al.*, 1995), and 1 in 500 for 9H10 (anti-hnRNP A1). Primary antibodies were visualized with FITC- or Texas red-conjugated goat anti-mouse or goat anti-rabbit antibodies (10 µg/ml) diluted in PBS containing 1% non-fat dry milk. Embryos were examined by confocal laser scanning microscopy on a Bio-Rad MRC1000.

Electron microscopy

Mutant and control embryos were isolated from microdrop cultures and immediately fixed in 0.1 M phosphate buffer, pH 7.4, containing 2% glutaraldehyde for at least 12 h at 4°C. Embryo samples were then post-fixed in 1% osmium tetroxide for 1 h, dehydrated, and embedded in Spurr low-viscosity resin. Thin sections were cut and examined with a JEOL JEM-1200EX II electron microscope.

BrdU incorporation in cultured blastocysts and DNA content analysis

Embryos were cultured in the presence of 20 µM BrdU for 18–24 h, and then fixed with methanol:acetone (1:1) for 10 min at room temperature (Pagano *et al.*, 1994). After three washes with PBS, embryos were treated with 1.5 N HCl for 10 min at room temperature. After four washes with PBS, embryos were incubated with PBS containing 1% non-fat dry milk for 15 min, and then with anti-BrdU antibodies (undiluted; Amersham, Life Science) for 1 h at room temperature. Anti-BrdU antibody binding was detected with FITC-conjugated goat anti-mouse antibodies (1 in 50 dilution; Amersham, Life Science). Embryos were stained for DNA in PBS containing 100 µg/ml propidium iodide for 60 min at room temperature. After three washes in PBS, embryos were embedded in Vectashield mounting medium, and examined by confocal laser scanning microscopy. The DNA content of individual cells of the scanned embryos was determined by cytometric quantitation of free-lying embryonic nuclei.

Import assay on whole mount embryos

The *in vitro* import assay was done essentially as described (Adam *et al.*, 1990). In brief, two (or occasionally four) embryos cultured in DMEM were rinsed once in transport buffer (20 mM HEPES, pH 7.5, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM dithiothreitol and 1 µg/ml of aprotinin, leupeptin, and pepstatin), followed by permeabilization in 80 µg/ml digitonin (Calbiochem; diluted from a 20 mg/ml stock solution in DMSO) in transport buffer on ice for 10 min. After one rinse in transport buffer, the embryos were transferred to 5 µl complete transport mixture which contained 40% reticulocyte lysate, 250 nM APC–NLS (Adam *et al.*, 1990) or unmodified APC as negative control (Calbiochem, San Diego, CA), 1 mM ATP, 10 mM creatine phosphate (Calbiochem), and 20 units/ml creatine phosphokinase (Calbiochem) in transport buffer. Import reactions were carried out in the dark for 5 or 10 min in a humidified chamber at room temperature and terminated by rinsing briefly in transport buffer. The embryos were then immediately examined by epifluorescence (Olympus BX50 microscope) and photographs were taken with a Kodak Royal Gold 1000 film. All embryos were photographed under identical conditions (exposures of 10 s). The concentration of APC–NLS in embryonic nuclei was quantitated by scanning the photographs with a densitometer (X-Rite 810) and deriving the values from a standard curve. On average, 12–18 free-lying nuclei were scanned per embryo. We determined the average nuclear concentration of APC–NLS of each *CAN*+/– and control embryo within the same reaction and compared them. Average nuclear concentrations of APC–NLS of two control embryos within the same import reaction were not significantly different (n=10 reactions; P<0.05).

In situ hybridization on whole mount embryos

In vitro cultured blastocysts were collected and washed with PBS. The embryos were then fixed in 4% paraformaldehyde (in PBS) for 15 min at 4°C, washed three times in PBS/0.1% Tween-20 (v/v; PBT), and permeabilized in RIPA buffer [50 mM Tris–HCl, pH 8, 150 mM NaCl, 1% Nonidet P-40 (v/v), 0.5% deoxycholate (w/v), 0.1% SDS (w/v)]. After three washes with PBT and one wash with 2× SSC, the embryos were hybridized with an oligo(dT) probe directly coupled to FITC as described (Amberg *et al.*, 1992).

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References

Adam,E.J.H. and Adam,S.A. (1994) Identification of cytosolic factors required for nuclear location sequence-mediated binding to the nuclear envelope. *J. Cell Biol.*, **125**, 547–555.

Adam,S.A. and Gerace,L. (1991) Cytosolic proteins that specifically bind nuclear localization signals are receptors for nuclear import. *Cell*, **66**, 837–847.

Adam,S.A., Marr,R.S. and Gerace,L. (1990) Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. *J. Cell Biol.*, **111**, 807–816.

Amberg,D.C., Goldstein,A.L. and Cole,C.N. (1992) Isolation and characterization of *RAT1*: an essential gene of *Saccharomyces cerevisiae* required for the efficient nucleocytoplasmic trafficking of mRNA. *Genes Dev.*, **6**, 1173–1189.

Amon,A., Irniger,S. and Nasmyth,K. (1994) Closing the cell cycle circle in yeast: G2 cyclin proteolysis initiated at mitosis persists until the activation of G1 cyclins in the next cycle. *Cell*, **77**, 1037–1050.

Bogerd,H.P., Fridell,R.A., Madore,S. and Cullen,B.R. (1995) Identification of a novel cellular cofactor for the Rev/Rex class of retroviral regulatory proteins. *Cell*, **82**, 485–494.

Chi,N.C., Adam,E.J.H. and Adam,S.A. (1995) Sequence and characterization of cytoplasmic nuclear protein import factor p97. *J. Cell Biol.*, **130**, 265–274.

Davis,L.I. (1995) The nuclear pore complex. *Annu. Rev. Biochem.*, **64**, 865–896.

Davis,L.I. and Blobel,G. (1986) Identification and characterization of a nuclear pore complex protein. *Cell*, **45**, 699–709.

Doye,V. and Hurt,E.C. (1995) Genetic approaches to nuclear pore structure and function. *Trends Genet.*, **11**, 235–241.

Doye,V., Wepf,R. and Hurt,E.C. (1994) A novel nuclear pore protein Nup133p with distinct roles in poly(A)$^+$ RNA transport and nuclear pore distribution. *EMBO J.*, **13**, 6062–6075.

Fischer,U., Huber,J., Boelens,W.C., Mattaj,I.W. and Lührmann,R. (1995) The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. *Cell*, **82**, 475–485.

Fornerod,M., Boer,J., van Baal,S., Jaeglé,M., Von Lindern,M., Murti,K.G., Davis,D., Bonten,J., Buijs,A. and Grosveld,G. (1995) Relocation of the carboxyterminal part of CAN from the nuclear envelope to the nucleus as a result of leukemia-specific chromosome rearrangements. *Oncogene*, **10**, 1739–1748.

Fritz,C.C., Zapp,M.L. and Green,M.R. (1995) A human nucleoporin-like protein that specifically interacts with HIV Rev. *Nature*, **376**, 530–533.

Gerace,L. (1995) Nuclear export signals and the fast track to the cytoplasm. *Cell*, **82**, 341–344.

Görlich,D. and Mattaj,I. (1996) Nucleocytoplasmic transport. *Science*, **271**, 1513–1518.

Görlich,D., Prehn,S., Laskey,R.A. and Hartmann,E. (1994) Isolation of a protein that is essential for the first step of nuclear protein import. *Cell*, **79**, 767–778.

Görlich,D., Vogel,F., Mills,A.D., Hartmann,E. and Laskey,R.A. (1995) Distinct functions for the two importin subunits in nuclear protein import. *Nature*, **377**, 246–248.

Gorsch,L.C., Dockendorff,T.C. and Cole,C.N. (1995) Conditional allele of the novel repeat-containing yeast nucleoporin *RAT7/NUP159* causes both rapid cessation of mRNA export and reversible clustering of nuclear pore complexes. *J. Cell Biol.*, **129**, 939–955.

Hogan,B., Beddington,R., Constantini,F. and Lacy,E. (1994) *Manipulating the Mouse Embryo: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Hsu,C. (1979) *In vitro* development of individually cultured whole mouse embryos from blastocyst to early somite stage. *Dev. Biol.*, **68**, 453–461.

Hurt,E.C. (1996) Importins/Karyopherins meet nucleoporins. *Cell*, **84**, 509–515.

Izaurralde,E. and Mattaj,I.W. (1995) RNA export. *Cell*, **81**, 153–159.

Kidder,G.M. (1992) The genetic program for preimplantation development. *Dev. Genet.*, **13**, 319–325.

Kraemer,D., Wozniak,R.W., Blobel,G. and Radu,A. (1994) The human can protein, a putative oncogene product associated with myeloid leukemogenesis, is a nuclear pore complex protein that faces the cytoplasm. *Proc. Natl Acad. Sci. USA*, **91**, 1519–1523.

Li,O., Heath,C.V., Amberg,D.C., Dockendorff,T.C., Copeland,C.S., Snyder,M. and Cole,C.N. (1995) Mutation or deletion of the *Saccharomyces cerevisiae RAT3/NUP133* gene causes temperature-dependent nuclear accumulation of poly(A)$^+$ RNA and constitutive clustering of nuclear pore complexes. *Mol. Cell. Biol.*, **6**, 401–417.

Loeb,J., Schlenstedt,G., Pellman,D., Kornitzer,D., Silver,P.A. and Fink,G.R. (1995) The yeast nuclear import receptor is required for mitosis. *Proc. Natl Acad. Sci. USA*, **92**, 7647–7651.

Melchior,F., Paschal,B., Evans,J. and Gerace,L. (1993) Inhibition of nuclear protein import by nonhydrolyzable analogues of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor *J. Cell Biol.*, **123**, 1649–1659. [Published erratum appears in *J. Cell Biol.*, **124**, 217 (1994).]

Michael,W.M., Choi,M. and Dreyfuss,G. (1995) A nuclear export signal in hnRNP A1: a signal-mediated, temperature-dependent nuclear protein export pathway. *Cell*, **83**, 415–422.

Moore,M.S. and Blobel,G. (1993) The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. *Nature*, **365**, 661–663.

Moroianu,J., Hijikata,M., Blobel,G. and Radu,A. (1995a) Mammalian karyopherin $\alpha_1\beta$ and $\alpha_2\beta$ heterodimers: α_1 or α_2 subunit binds nuclear localization signal and β subunit interacts with peptide repeat-containing nucleoporins. *Proc. Natl Acad. Sci. USA*, **92**, 6532–6536.

Moroianu,J., Blobel,G. and Radu,A. (1995b) Previously identified protein of uncertain function is karyopherin α and together with karyopherin β docks import substrate at nuclear pore complexes. *Proc. Natl Acad. Sci. USA*, **92**, 2008–2011.

Mortensen,R.M., Conner,D.A., Chao,S., Geisterfer-Lowrance,A.A.T. and Seidman,J.G. (1992) Production of homozygous mutant ES cells with a single targeting construct. *Mol. Cell. Biol.*, **12**, 2391–2395.

Mountford,P.S. and Smith,A.G. (1995) Internal ribosome entry sites and dicistronic RNAs in mammalian transgenesis. *Trends Genet.*, **11**, 179–184.

Pagano,M., Theodoras,A.M., Tam,S.W. and Draetta,G.F. (1994) Cyclin-D1-mediated inhibition of repair and replicative DNA synthesis in human fibroblasts. *Genes Dev.*, **8**, 1627–1639.

Panté,N. and Aebi,U. (1994) Toward the molecular details of the nuclear pore complex. *J. Struct. Biol.*, **113**, 179–189.

Piñol-Roma,S. and Dreyfuss,G. (1992) Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm. *Nature*, **355**, 730–732.

Radu,A., Blobel,G. and Moore,M.S. (1995a) Identification of a protein complex that is required for nuclear protein import and mediates docking of import substrate to distinct nucleoporins. *Proc. Natl Acad. Sci. USA*, **92**, 1769–1773.

Radu,A., Moore,M.S. and Blobel,G. (1995b) The peptide repeat domain of nucleoporin Nup98 functions as a docking site in transport across the nuclear pore complex. *Cell*, **81**, 215–222.

Rexach,M. and Blobel,G. (1995) Protein import into nuclei: association and dissociation reactions involving transport substrate, transport factors and nucleoporins. *Cell*, **83**, 683–692.

Rout,M.P. and Wente,S.R. (1994) Pores for thought: nuclear pore complex proteins. *Trends Biochem. Sci.*, **4**, 357–363.

Siniossoglou,S., Wimmer,C., Rieger,M., Doye,V., Tekotte,H., Weise,C., Emig,S., Segref,A. and Hurt,E.C. (1996) A novel complex of nucleoporins, which includes Sec13p and a Sec13p homolog, is essential for normal nuclear pores. *Cell*, **84**, 265–275.

Siomi,H. and Dreyfuss,G. (1995) A nuclear localization domain in the hnRNP A1 protein. *J. Cell Biol.*, **129**, 551–550.

Stutz,F., Neville,M. and Rosbash,M. (1995) Identification of a novel nuclear pore-associated protein as a functional target of the HIV-1 Rev protein in yeast. *Cell*, **82**, 495–506.

Tobey,T.A., Oishu,N. and Crissman,H.A. (1990) Cell cycle synchronization: reversible induction of G_2 synchrony in cultured rodent and human diploid fibroblasts. *Proc. Natl Acad. Sci. USA*, **87**, 5104–5108.

van Deursen,J. and Wieringa,B. (1992) Targeting of the creatine kinase M gene in embryonic stem cells using isogenic and nonisogenic vectors. *Nucleic Acids Res.*, **20**, 3815–3820.

van Deursen,J., Heerschap,A., Oerlemans,F., Ruitenbeek,W., Jap,P., ter Laak,H. and Wieringa,B. (1993) Skeletal muscles of mice deficient in M-CK lack burst activity. *Cell*, **74**, 621–631.

von Lindern,M., Fornerod,M., van Baal,S., Jaeglé,M., de Wit,T., Buijs,A. and Grosveld,G. (1992) The translocation (6;9), associated with a specific subtype of acute myeloid leukemia, results in the fusion of two genes, *dek* and *can*, and the expression of a chimeric, leukemia-specific *dek-can* mRNA. *Mol. Cell. Biol.*, **12**, 1687–1697.

Wen,W., Meinkoth,J.L., Tsien,R.Y. and Taylor,S.S. (1995) Identification of a signal for rapid export of proteins from the nucleus. *Cell*, **82**, 463–473.

Wente,S.R. and Blobel,G. (1993) A temperature-sensitive *NUP116* null mutant forms a nuclear envelope seal over the yeast nuclear pore complex thereby blocking nucleo-cytoplasmic traffic. *J. Cell. Biol.*, **123**, 275–284.

Wente,S.R. and Blobel,G. (1994) *NUP145* encodes a novel yeast glycine-leucine-phenylalanine- glycine (GLFG) nucleoporin required for nuclear envelope structure. *J. Cell Biol.*, **125**, 955–969.

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3 Overexpression of the nucleoporin CAN/NUP214 induces growth arrest, nucleocytoplasmic transport defects and apoptosis

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Abstract

The human *CAN* gene was first identified as a target of t(6;9)(p23;q34), associated with acute myeloid leukemia and myelodysplastic syndrome, which results in the expression of a *DEK-CAN* fusion gene. CAN, also called NUP214, is a nuclear pore complex protein that contains multiple FG-peptide sequence motifs. It exists in a subcomplex at the NPC with at least two other proteins, hCRM1 and NUP88. Depletion of CAN in knock-out mouse embryonic cells results in cell cycle arrest in G_2, followed by inhibition of nuclear protein import and a block of mRNA export. We overexpressed CAN and DEK-CAN in U937 myeloid precursor cells. DEK-CAN expression did not interfere with terminal myeloid differentiation of U937 cells, whereas CAN-overexpressing cells arrested in G_0, accumulated mRNA in their nuclei, and died in an apoptotic manner. Interestingly, we found that hCRM1 and import factor p97/importin β colocalized with the ectopically expressed CAN protein, resulting in depletion of both factors from the NPC. Overexpression of CAN's C-terminal FG-repeat region, which contains the binding site for hCRM1, caused sequestering of hCRM1 in the nucleoplasm and was sufficient to inhibit cell growth and to induce apoptosis. These results confirm that CAN plays a crucial role in nucleocytoplasmic transport and imply an essential role for hCRM1 in cell growth and survival.

Introduction

The recurrent chromosome translocation (6;9)(p23;q34), found in acute myeloid leukemia (AML) and myelodysplastic syndrome, fuses together the coding regions of two genes, *DEK* and *CAN* (Von Lindern et al., 1992). The resulting *DEK-CAN* mRNA encodes an in-frame chimeric protein that contains almost the entire DEK protein linked to the C-terminal two-thirds of CAN. DEK is a nuclear DNA-binding protein (Fu et al., 1997). CAN, also called NUP214, is a nuclear pore complex (NPC) component, or nucleoporin, and contains NPC protein-specific FG-repeat sequences (Fornerod et al., 1995; Kraemer et al., 1994). Its deletion in mouse embryos results in cell cycle arrest in G_2, followed by a block in mRNA export and inhibition of nuclear protein import (van Deursen et al., 1996).

The central region of CAN contains two predicted coiled-coil domains and anchors the protein to the NPC. Approximately the same domain binds NUP88, a novel NPC component of 88 kDa (Fornerod et al., 1996; Fornerod et al., 1997). The translocation breakpoint in CAN lies in the middle of this region, and in the DEK-CAN fusion protein both nuclear envelope localization and NUP88 binding are disrupted (Fornerod et al., 1996). DEK-CAN is nuclear and relocation of the C-terminus of CAN from the nuclear envelope to the nucleus may contribute to the leukemogenic potential of the fusion protein (Fornerod et al., 1995).The C-terminus of CAN, consisting of nucleoporin-specific repeats, can direct the protein to the nucleus in the absence of the NPC-binding domain (Fornerod et al., 1995). This relocation is mediated by binding of this part of the repeat, which is present in CAN as well as in DEK-CAN, to hCRM1, a recently identified human protein of 112 kDa that moves from the NPC to the nucleus (Fornerod et al., 1996; Fornerod et al., 1997). hCRM1 shows considerable homology to yeast CRM1, a protein required for correct chromosome structure. Its dynamic localization, coupled to the discovery that hCRM1 is a member of a newly identified family of NPC-interacting proteins, suggests that hCRM1 is a nucleocytoplasmic transport factor (Fornerod et al., 1997).

The NPC is a supramolecular structure that contains multiple copies of about 100 different proteins. It mediates bidirectional transport of macromolecules between the cytoplasm and the nucleus (reviewed by Davis, 1995; Fabre and Hurt, 1994; Panté and Aebi, 1994; Rout and Wente, 1994). Karyophilic proteins contain a nuclear localization signal (NLS) that is recognized by the NLS-receptor, also called importin α, Srp1p, or karyopherin α (Adam and Adam, 1994; Adam and Gerace, 1991; Görlich et al., 1994; Moroianu et al., 1995a; Weis et al., 1995). This complex docks to the cytoplasmic side of the NPC via p97, synonymous with importin β and karyopherin β (Chi et al., 1995; Görlich et al., 1995; Radu et al., 1995a). p97 binds to repeat-containing nucleoporins *in vitro* and *in vivo* (Iovine et al., 1995; Kraemer et al., 1995; Radu et al., 1995a; Radu

et al., 1995b; Rexach and Blobel, 1995). After this initial docking step, the complex is translocated through the pore via an energy-dependent process that is mediated by the small nuclear GTPase Ran and its cofactors (Melchior et al., 1993; Moore and Blobel, 1993). At the nuclear side of the NPC, the NLS-receptor and the substrate are released into the nucleoplasm, while p97 remains bound to the NPC (Görlich et al., 1995; Moroianu et al., 1995b). Protein and ribonucleoprotein (RNP) export from the nucleus also occurs via a receptor-mediated, energy-dependent mechanism and nuclear export signals (NESs), identified in a number of proteins, are thought to play a role in this process (for reviews see Gerace, 1995; Görlich and Mattaj, 1996; Izaurralde and Mattaj, 1995). Importin α was recently found to be involved in the export of capped RNA polymerase II transcripts from the nucleus, and dissociation of the importin α-RNA complex into the cytoplasm is mediated by importin β (Görlich et al., 1996).

Depletion of nuclear pore components often leads to nucleocytoplasmic transport defects, growth inhibition and cell death. Such effects have been demonstrated for several yeast mutants (Doye and Hurt, 1995; Rout and Wente, 1994), and recently for CAN/NUP214 in the mouse (van Deursen et al., 1996). The yeast studies have also shown that it is important to maintain the correct relative stoichiometry of NPC components, as overexpression of some components severely restricts cell growth (Davis and Fink, 1990; Wente and Blobel, 1993; Wozniak et al., 1994). Overexpression of CAN and DEK-CAN in cell lines has proven to be toxic (Fornerod et al., 1995). To address why overexpression of these proteins is cytotoxic and to study the effects of overexpression on myeloid differentiation, we introduced inducible CAN and DEK-CAN genes into the human myeloid precursor U937 cells. Expression of the AML-specific DEK-CAN protein did not affect differentiation of U937 cells, whereas overexpression of CAN in U937 cells arrested them in G_0, caused a defect in mRNA export and mislocalization of hCRM1 and p97, and ultimately led to apoptosis. Overexpression of the hCRM1-binding domain of CAN resulted in nuclear sequestering of hCRM1 and was sufficient to inhibit cell growth and to induce cell death.

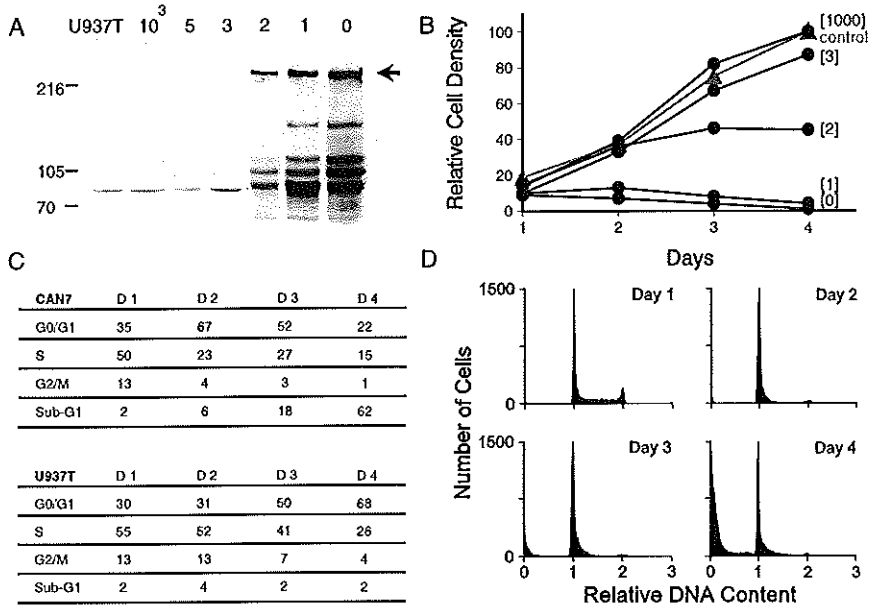
Results

CAN overexpression induces G_0 arrest and apoptosis

To study the effects of CAN and DEK-CAN on growth, survival and differentiation of myeloid cells, we introduced inducible *CAN* and *DEK-CAN* genes into the human myeloid precursor U937 cells. First, we generated cell line U937T, which expressed tetVP16 in a tetracycline-dependent manner (Gossen and Bujard, 1992; Shockett et al., 1995; Vignali et al., 1996) but maintained normal growth characteristics. Subsequently,

this clone was stably transfected with expression constructs containing HA1-epitope tagged (tt) CAN or DEK-CAN cDNAs under the control of the tetVP16-responsive promoter. Each cell line used for this study was selected from a number of clonal lines based on the relative expression level of the transfected gene after tetracycline withdrawal.

A clone that expressed relatively high levels of ttCAN upon withdrawal of



CAN7	D 1	D 2	D 3	D 4
G0/G1	35	67	52	22
S	50	23	27	15
G2/M	13	4	3	1
Sub-G1	2	6	18	62

U937T	D 1	D 2	D 3	D 4
G0/G1	30	31	50	68
S	55	52	41	26
G2/M	13	13	7	4
Sub-G1	2	4	2	2

Figure 1. CAN-overexpressing cells are growth inhibited

(A) Inducible expression of HA1-tagged CAN in CAN7 cells grown for 24 hr in the presence of 1000, 5, 3, 2, 1, and 0 ng/ml of tetracycline, as indicated above the lanes, was assayed by Western blot analysis of the 6 % SDS-PAGE gel. Induced parental U937T cells serve as a negative control. Each lane contains lysate from $5x10^5$ cells. The blot was probed with the anti-HA1 monoclonal 12CA5. The arrow indicates ttCAN protein. Lysates of cells expressing high amounts of ttCAN protein show specific degradation products. The sizes of molecular weight standards, run in an adjacent lane, are indicated on the left in kilodalton. (B) Growth curves of induced CAN7 cells (●) and U937T control cells (▲). Cultures were maintained in medium containing the indicated tetracycline concentrations and viability was measured daily by using a non-radioactive proliferation assay. The relative density was calculated as a percentage of the density of uninduced cells on day 4. Mean values of triplicates are plotted, the standard deviations were below 10 %. This experiment is one of three that all gave similar results. (C) Cell cycle phase distribution of induced CAN7 cells (upper panel) and U937T cells (lower panel) at 1, 2, 3, and 4 days following tetracycline withdrawal was calculated from flow cytometric measurements of DNA content. (D) Flow cytometry profiles showing DNA fluorescence of propidium iodide-stained CAN7 cell nuclei at 1, 2, 3, and 4 days following withdrawal of tetracycline. This is a representative experiment of three, all of which gave similar results.

tetracycline, CAN7, was selected to study the effects on cell growth of overexpression of different amounts of CAN by varying the concentrations of tetracycline in the culture medium. ttCAN was detected by immunofluorescence staining, using the anti-HA1 monoclonal antibody, as early as 10 hr after induction and reached maximal levels after ~24 hr (data not shown). Western blot analysis showed that low concentrations of tetracycline in the culture medium (1 and 2 ng/ml) allowed expression of intermediate levels of ttCAN, whereas complete absence of tetracycline caused greater amounts of ttCAN to accumulate (Fig. 1A). In the presence of 3 ng/ml tetracycline, ttCAN was barely detectable on Western blot, whereas no protein was detected in lysates from cells cultured at higher tetracycline concentrations. The growth curves of induced and uninduced CAN7 cells showed that growth inhibition was directly proportional to the

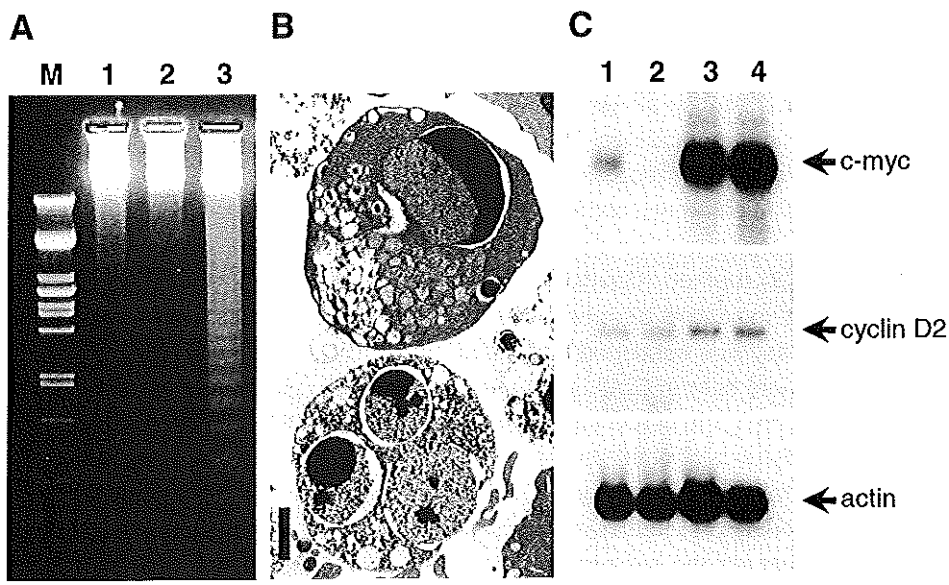


Figure 2. Overexpression of CAN induces G_0 apoptosis in U937T cells

(A) Agarose gel electrophoresis of DNA from CAN7 cells cultured for three days in the absence of tetracycline (lane 3) showed internucleosomal DNA cleavage, whereas DNA from the parent U937T cells grown in the presence (lane 1) or absence (lane 2) of 1 µg/ml tetracycline remained unfragmented. Pst1-digested λ DNA served as a molecular weight marker (lane M). (B) Electron micrographs showing apoptotic CAN-overexpressing CAN7 cells after three days of induction. The bar represents 2 µm. (C) Northern blot analysis of 15 µg RNA isolated from the total culture (lane 1) and the FACS-sorted diploid cell fraction (lane 2) of CAN7 cells induced for 40 hr, compared to the total cultures of uninduced CAN7 cells (lane 3) and induced parental U937T cells (lane 4). The amounts of *c-myc* (2.4 kb; upper panel), and *cyclin* D_2 (6.0 kb; middle panel) mRNA were compared to the levels of *actin* mRNA (2.0 kb; lower panel).

amount of expressed ttCAN and inversely proportional to the tetracycline concentration in the medium (Fig. 1B). Like the parental U937T cells, the uninduced CAN7 cells continued to grow with a doubling time of about 24 hr, whereas cells expressing the highest levels of ttCAN (0 and 1 ng tetracycline/ml) stopped growing and their numbers were reduced after three days. Under conditions that gave expression of intermediate levels of ttCAN protein (2 ng/ml tetracycline), we observed some increase in cell number, albeit at a significantly lower rate than without induction.

To examine if fully induced CAN7 cells arrested at a particular phase of the cell cycle, we studied the DNA content of the overexpressing cells by FACS analysis of propidium iodide-stained nuclei (Fig. 1C and D). After two days of CAN induction, the percentage of cells in S phase was reduced from 13 % to 4 %, and the percentage of cells in G_2/M from 50 % to 23 %. The percentage of cells with a diploid DNA content was increased from 35 % to 67 %, suggesting that the cells had arrested at G_1/G_0 . At this time point very few cells were scored with a sub-G_1 DNA content (see below). By day 3, however, 18 % of the cells showed a sub-G_1 DNA content and this fraction increased to 62 % by day 4 after tetracycline withdrawal (Fig. 1C, upper panel). The increase in the percentage of hypodiploid nuclei in ttCAN-overexpressing cells indicated that the cells became apoptotic (Nicoletti et al., 1991). Moreover, the cells displayed morphologic features of apoptosis, including DNA fragmentation (Fig. 2A), nuclear segmentation, and chromatin condensation (Fig. 2B). Cells also stained positively with the terminal-deoxytransferase-mediated deoxyuridine triphosphate-biotin nick end-labeling (TUNEL) method (Gavrieli et al., 1992), which detects double strand DNA breaks that are indicative of apoptosis (data not shown). U937T cells grown in the absence of tetracycline continued to cycle normally. They reached confluency by day 4, resulting in more cells in G_1/G_0 and fewer cells in S and G_2/M. In contrast to ttCAN-overexpressing cells, the percentage of cells with a sub-G_1 DNA content remained low, between 2 % and 4 % (Fig. 1C, lower panel).

To determine if CAN-arrested CAN7 cells were blocked in G_1 or G_0, we studied their *c-myc* mRNA expression levels. Cycling cells express high levels of *c-myc* mRNA, whereas cells that exit the cell cycle and arrest in G_0 do not transcribe *c-myc* (Kelly and Siebenlist, 1986). CAN7 cells were induced in the absence of tetracycline for 40 hr, and RNA was isolated from the total culture and from cells sorted for a diploid DNA content. RNA from total cultures of uninduced CAN7 cells and parental U937T cells was isolated to serve as a control. Northern blot analysis showed a dramatic decrease in the amount of *c-myc* mRNA in the ttCAN expressing cells compared to that expressed in uninduced cells. The low level of *c-myc* mRNA in the total culture was derived from the fraction of cells that remained cycling, since the sorted diploid cell fraction was negative for *c-myc* mRNA (Fig. 2C, upper panel). A similar, albeit smaller, reduction was observed in the amounts of *cyclin* D_2 mRNA (Fig. 2C, middle panel), which is normally present throughout the cell cycle (Sherr, 1996). The more rapid downregulation of *c-myc* could

be caused by the very short half-life of *c-myc* mRNA (Waters et al., 1991).These data strongly suggest that, prior to apoptosis, the ttCAN-overexpressing cells exited the cell cycle and arrested in G_0.

Bcl-X_L co-expression does not prevent apoptosis

Bcl-X_L, a member of the Bcl-2 family, can protect cells against a variety of apoptosis inducers (Boise et al., 1993; González-García et al., 1995; Gottschalk et al., 1996). We examined whether constitutive co-expression of the Bcl-X_L gene with ttCAN in CAN7 cells could inhibit CAN-induced apoptosis. A representative stably transfected CAN7 clone, CAN7-B2, expressed high levels of Bcl-X_L protein (Fig. 3A) and grew slower than the parental CAN7 clone (Fig. 3). This could be a direct consequence of Bcl-X_L expression, since Bcl-2 expression has been shown to prolong the G_1 phase in U937 and other cell lines (Borner, 1996). Bcl-X_L expression had no influence on growth inhibition or cell death after tetracycline was removed from several independent CAN7-Bcl-X_L clones (Fig. 3), indicating that Bcl-X_L could not inhibit the apoptotic cell death of induced CAN7 cells.

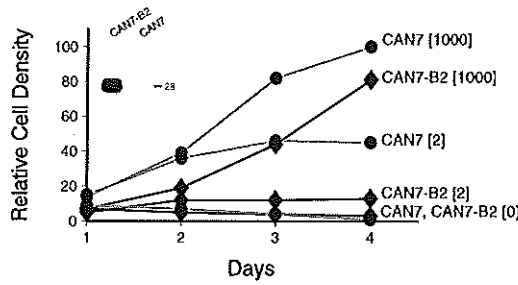


Figure 3. Bcl-X_L co-expression does not rescue CAN-overexpressing cells

Growth curves of a representative Bcl-X_L-overexpressing CAN7 clone, CAN7-B2 (◆), compared to CAN7 (●), both grown in 1000, 2 and 0 ng/ml tetracycline for four days. Mean relative density values of triplicate cultures are plotted against time, the standard deviations were below 10 %. This experiment is one of three, all of which gave similar results. Inset: Western blot of a 9 % SDS-PAGE gel containing lysate from $5x10^5$ cells per lane, probed with a mouse monoclonal antibody to Bcl-X_L. The 29 kDa doublet represents the Bcl-X_L protein. The position of the 28 kDa molecular weight standard is indicated on the right.

Transport defects in CAN-overexpressing cells

We studied the subcellular localization of CAN in induced and uninduced cells by indirect immunofluorescence with the polyclonal CAN antiserum αCNC (Fornerod et al., 1995). In the parental U937T cells, endogenous CAN levels were low and the protein localized to the nuclear envelope (Fig. 4A). During the first day after tetracycline withdrawal, ttCAN in CAN7 cells mainly localized to the nuclear envelope and cytoplasmic speckles. The latter structures could be annulate lamellae or simply aggregates of insoluble protein (Fig. 4B). However, during the second day after induction, an increasing percentage of cells showed nuclear localization of ttCAN and, by the third day, 90% of the cells had ttCAN in the nucleus (Fig. 4C). The nuclear staining was diffuse, with a few strong dots. These results demonstrate that the loss of cell viability upon ttCAN overexpression coincides with the accumulation of ttCAN in the nucleoplasm.

Because CAN is a nucleoporin, we asked whether the cytotoxity of its overexpression coincided with perturbations in its NPC transport function. We measured the nuclear export capacity of the ttCAN-overexpressing cells by monitoring the localization of polyadenylated RNA by *in situ* hybridization with an oligo$(dT)_{50}$ probe directly coupled to FITC (Amberg et al., 1992). Forty-eight hours after

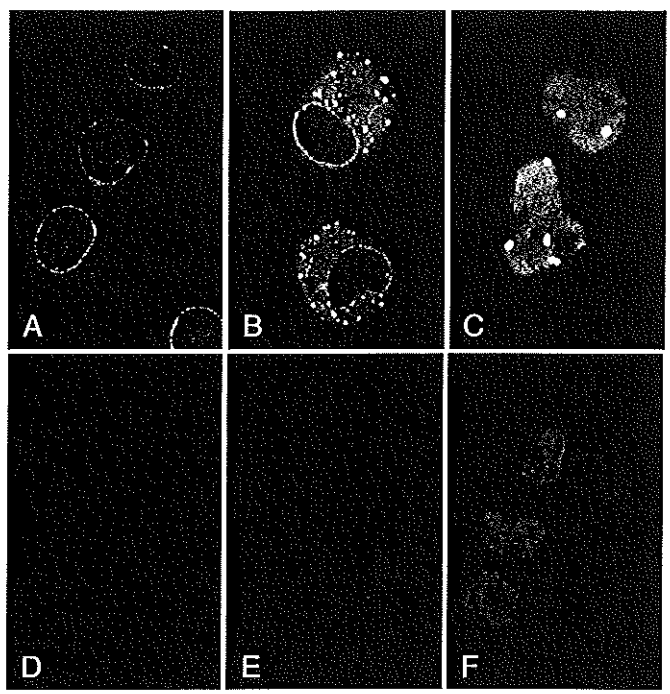


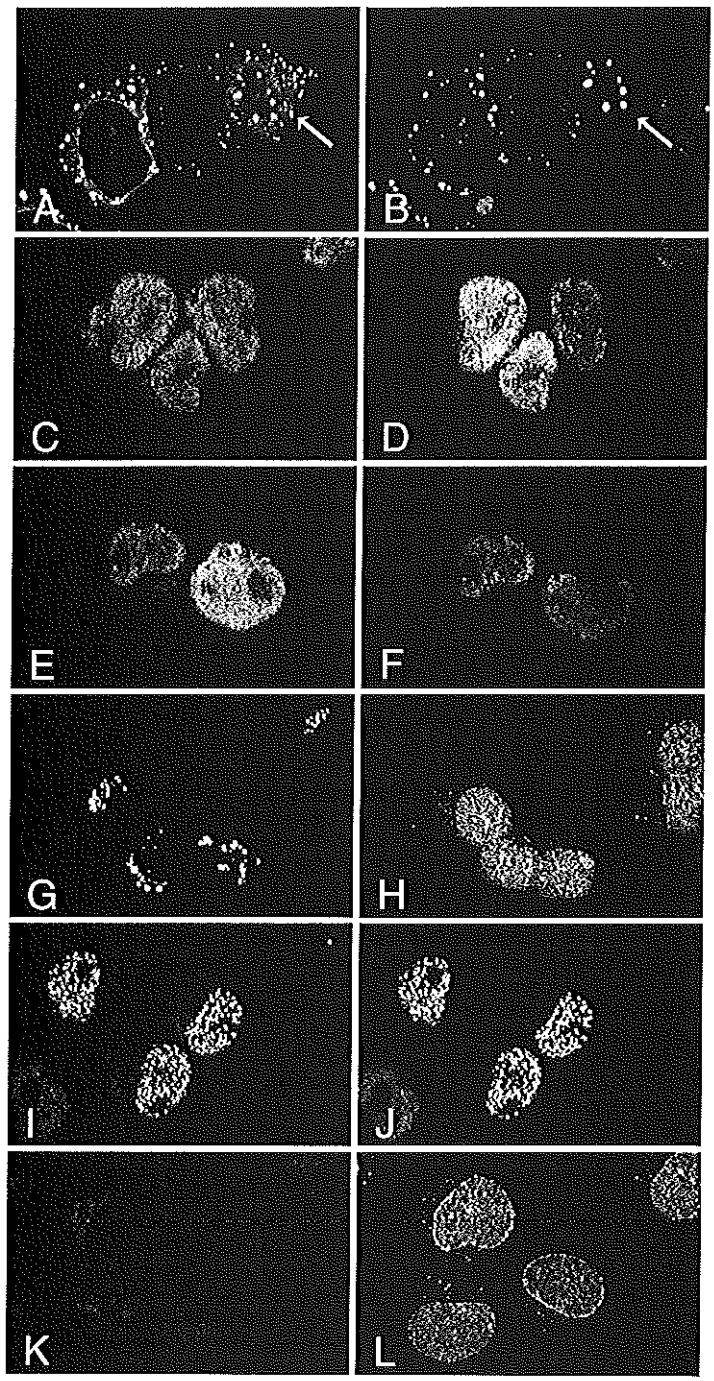
Figure 4. Polyadenylated RNA export defect in CAN-overexpressing cells

Confocal images of endogenous CAN expression in U937T cells (A) and overexpressed CAN in CAN7 cells 20 hr (B) and 48 hr (C) after induction, stained with the anti-CAN antiserum αCNC. Subcellular localization of polyadenylated RNA in control U937T cells (D) and CAN7 cells after 48 hr (E) and 60 hr (F) of induction analyzed by *in situ* hybridization with an FITC-conjugated oligo $(dT)_{50}$ probe.

tetracycline withdrawal, poly(A)$^+$ RNA in both U937T cells (Fig. 4D) and in CAN7 cells (Fig. 4E) appeared to be diffusely distributed throughout the nucleoplasm and cytoplasm. In contrast, 56-60 hr after ttCAN induction only CAN7 cells showed strong nuclear staining, indicating nuclear accumulation of poly(A)$^+$ RNA (Fig. 4F). At this time point, most cells were still alive and arrested in G_0. As a control, we induced apoptosis by culturing U937T cells for 48 hr in the presence of 50 µg/ml of the protein synthesis-inhibitor cycloheximide. In this case, apoptosis was not preceded by mRNA accumulation in the nucleus (data not shown), suggesting that the block in mRNA export observed CAN7 cells was specific for CAN overexpression.

The nuclear protein import capacity of induced CAN7 cells was assessed *in vitro* by examining the accumulation of an NLS-linked fluorescent substrate, APC-NLS, in the nuclei of digitonin-permeabilized cells (Adam et al., 1990). After 24 hr of tetracycline withdrawal, ttCAN-overexpressing CAN7 cells showed an import capacity similar to U937T cells, whereas CAN7 cells that had been induced for 48 hr did not appreciably import APC-NLS (not shown). These results indicated that the inhibition of nuclear protein import in CAN-overexpressing cells coincided with the cell cycle arrest. Therefore, we compared the import capacity of CAN7 cells two days after tetracycline withdrawal with U937T cells that had reached their maximal density four days after seeding. In both cultures almost 70 % of the cells were arrested with a diploid DNA content (Fig. 1C). Unexpectedly, we could not detect NLS protein import in the density-arrested U937T cells either, which makes it impossible to distinguish between cell cycle arrest and CAN overexpression as the primary cause for the import defect.

Inhibition of nucleocytoplasmic transport may be caused by gross structural alterations in the NPC or nuclear envelope or by functional perturbation. Thin section electron microscopy did not reveal structural perturbations of the NPC or nuclear envelope in CAN-overexpressing CAN7 cells after 3 days of induction (data not shown). Therefore, we assessed whether the toxicity of excess CAN resulted from functional inactivation of CAN-interacting proteins. hCRM1, a recently identified protein that co-immunoprecipitates with CAN, localizes to the nuclear envelope and the nucleus in normal cells and is thought to have a nuclear transport function (Fornerod et al., 1997). We used immunopurified polyclonal antiserum to detect hCRM1 (Fornerod et al., 1997) in combination with a monoclonal antibody, 12CA5, to the HA1-epitope to detect ttCAN in double immunostaining experiments. Strikingly, we found that hCRM1 colocalized with overexpressed CAN in the nuclear envelope, cytoplasm, and nucleus of CAN7 cells (Fig. 5A and B). In cells that expressed CAN in both cytoplasmic and nuclear speckles, hCRM1 preferentially colocalized with the nuclear structures (cell indicated by arrow in Fig. 5A and B). The accumulation of ttCAN in the nucleus of CAN7 cells after three days resulted in colocalization of hCRM1 in the nucleus and depletion of this factor from the nuclear envelope. We then studied the localization of the transport factor p97/importin β, which binds to CAN and other FG-repeat containing nucleoporins *in*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vitro and localizes to the NPC and cytoplasm (Chi et al., 1995; Görlich et al., 1995; Radu et al., 1995a). We immunostained induced CAN7 cells with a monoclonal antibody to p97 (Chi et al., 1995) and αCNC, and found that p97 colocalized with ectopically expressed CAN protein in the nuclear envelope and the cytoplasmic speckles. Two days after ttCAN induction, colocalization also occurred in the nucleus, which resulted in depletion of p97 from the nuclear envelope (Fig. 6A and B).

Expression of DEK-CAN and CAN mutants in U937 cells

To map the region of CAN that mediated cycle arrest and apoptosis, we studied the response of U937T cells to induced expression of CAN mutants and the leukemia-specific DEK-CAN fusion protein (Fig. 7). Clones were selected for high expression after induction, however, some mutants, including DEK-CAN, did not reach the expression levels of ttCAN (Fig. 8A). Most of the mutants described in this study were analyzed previously for subcellular localization and co-immunoprecipitating proteins in transient transfection studies (Fornerod et al., 1996). The N-terminus of CAN (CAN 1-1058) and a shorter central region (CAN 589-1058) both associate with the NPC and bind NUP88. Expression of these mutants did not affect cell growth or viability (Fig. 8B). However, expression of C-terminal regions of CAN, such as CAN 816-2090, which localizes to the NPC, cytoplasm, and nucleus, and the nuclear mutant CAN 1140-2090, inhibited cell growth (Fig. 8B). This effect could be attributed to overexpression of the most C-terminal FG-repeat-containing region (CAN 1864-2090), that was sufficient for the lethal phenotype (Fig. 8B). Importantly, this part of CAN harbors the hCRM1-binding domain. DNA histogram analysis (Fig. 8C and D) of induced CAN 1864-2090 cells showed that two days after tetracycline withdrawal the percentage of cells in S phase was reduced from 50 % to 25 %, and the percentage of cells in G_0/G_1 had increased from 36 % to 58 %. The percentage of cells with a sub-G_1 DNA content was 27 % after three days of induction, and 40 % after four days (Fig. 8C). This demonstrates that overexpression of the hCRM1- binding region is sufficient to cause cycle arrest and cell death. The

Figure 5. Colocalization of full-length CAN and CAN mutants with hCRM1

Subcellular distribution of hCRM1 and CAN proteins after two days of induction. (A, C, E, G, I, and K) Confocal microscopy showing immunodetection of induced CAN and mutant proteins by an anti-HA1 monoclonal antibody followed by a goat anti-mouse Texas Red-conjugated secondary antibody. (B, D, F, H, J, and L) Distribution of endogenous hCRM1 in the same cells stained with the α-hCRM1 antiserum and a goat anti-rabbit FITC-linked antibody. hCRM1 colocalized with full-length CAN (A and B), with the C-terminal FG-repeat regions of CAN, CAN 1864-2090 (C and D) and CAN (1140-1340, 1864-1912, 1984-2090) (E and F), and with DEK-CAN (I and J). In contrast, hCRM1 did not colocalize with the more N-terminally located FG-repeat region of CAN, represented by CAN 1558-1840 (G and H); it showed normal distribution in the nuclear membrane and the nucleoplasm, similar to that of induced U937T cells (K and L).

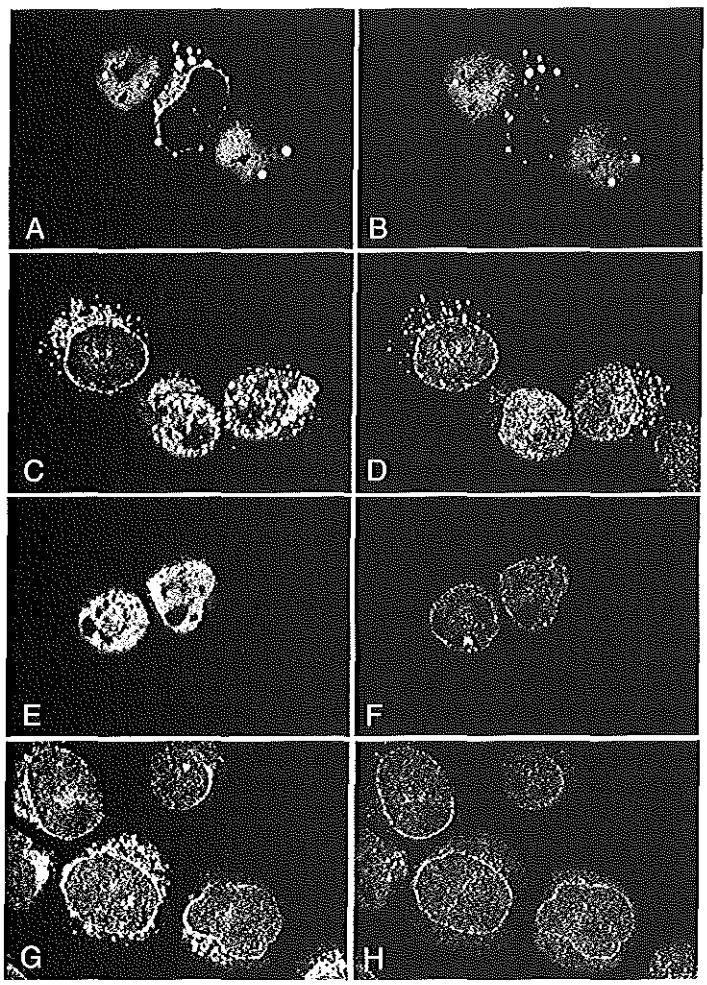


Figure 6. Double immunostaining of CAN and mutants with p97/importin β

Subcellular distribution of p97 and CAN proteins after two days of induction. (A, C, E, and G) Confocal images of indirect immunofluorescence with the anti-CAN polyclonal antiserum αCNC, detected with a Texas Red-conjugated goat anti-rabbit antibody. (B, D, F, and H) Confocal images of the same cells immunostained with an antibody to p97 (mAb3E9), detected with an FITC-conjugated goat anti-mouse antibody. Endogenous p97 colocalized with overexpressed CAN in the nuclear membrane and in the cytoplasmic and nucleoplasmic speckles (A and B). In cells overexpressing CAN 816-2090, only the nuclear membrane and the cytoplasmic speckles showed colocalization of the CAN mutant with p97 (C and D). Cells overexpressing CAN 1140-2090 showed normal p97 localization in the nuclear membrane (E and F), comparable to cells expressing endogenous levels of CAN (G and H).

effect was milder than when the full-length CAN is overexpressed, possibly because of subtle differences in the expression levels between the two clones. CAN deletion

mutants that lack part of the region necessary for hCRM1 co-immunoprecipitation (CAN (1140-1340, 1864-1912, 1984-2090) and CAN 1864-2017), still localized to the nucleus, and exhibited the lethal phenotype albeit at considerably higher expression levels (Fig. 8A and B). The FG-repeat-containing region just N-terminal of the hCRM1-binding region (CAN 1558-1840), was cytoplasmic and did not affect cell growth (Fig. 8B). The highest inducible DEK-CAN clone, DEK-CAN58, expressed only about 25% of the amount of CAN protein produced in CAN7, as estimated by Western blot analysis (Fig. 8A). This clone was slightly growth inhibited, but did not die from apoptosis (Fig. 8B).

Double immunofluorescence staining studies of CAN mutants (Fig. 5, left panel) with hCRM1 (Fig. 5, right panel) showed that endogenous hCRM1 colocalized with CAN 816-2090 in the cytoplasm and in the nucleus (not shown). The endogenous hCRM1 also colocalized with the hCRM1-binding domain (CAN 1864-2090) in the nucleus (Fig. 5C and D). The nuclear mutants CAN (1140-1340, 1864-1912, 1984-2090) (Fig.5E and F) and CAN 1864-2017 (not shown), which both lack part of the hCRM1-binding domain that is required to co-immunoprecipitation hCRM1 (Fornerod et al., 1996), still caused redistribution of hCRM1 to the nucleus. In contrast, CAN 1558-1840, which also does not coprecipitate hCRM1 (Fornerod et al., 1996), did not colocalize with hCRM1 (Fig. 5G and H) nor did it influence cell growth. Although DEK-CAN levels were not high enough to induce cell death, the staining patterns of DEK-CAN and hCRM1 were overlapping in high-expressing cells (Fig. 5I and J). Taken together, all (partly) nuclear CAN mutants colocalize with hCRM1, suggesting that the region of CAN needed for *in vivo* interaction with hCRM1 is smaller than the hCRM1-binding domain identified by co-immunoprecipitation. High expression levels of these mutants resulted in depletion of hCRM1 from the nuclear envelope, which coincided with growth inhibition and cell death. These data suggest that nuclear sequestration inhibits an essential transport function of hCRM1.

We studied the distribution of endogenous p97 (Fig. 6, right panel) in cells expressing CAN mutants (Fig. 6, left panel) and found colocalization of p97 with CAN 816-2090 in the nuclear envelope and the cytoplasmic speckles. Cells expressing this CAN mutant in nuclear speckles showed a normal p97 distribution (Fig. 6C and D). Expression of DEK-CAN (not shown) or CAN 1040-2090 (Fig. 6E and F) did not affect p97 localization, indicating that sequences in CAN's central region mediate this effect.

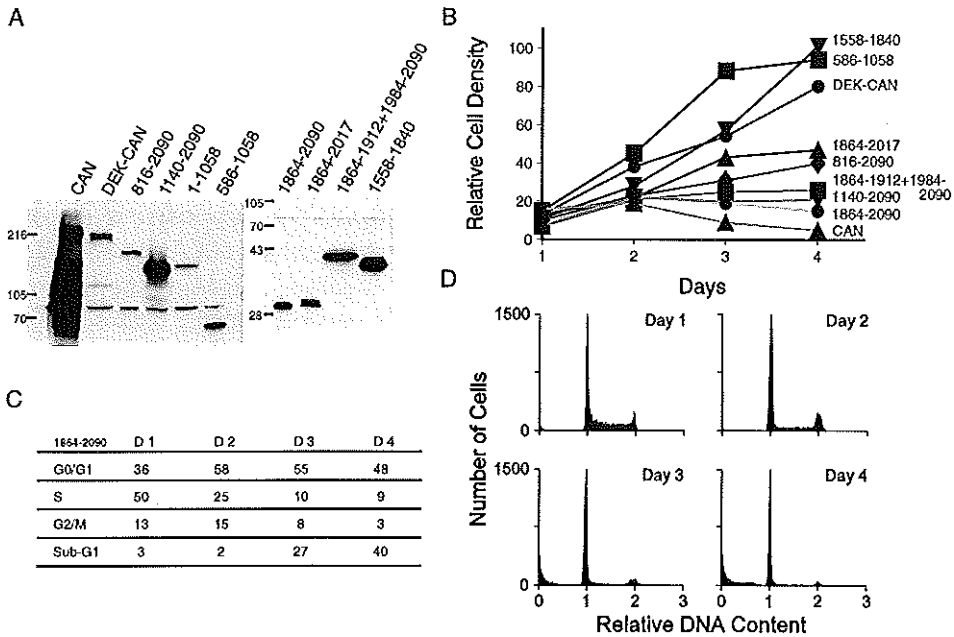
Poly(A)$^+$ RNA in clones expressing DEK-CAN or the C-terminus of CAN was diffusely distributed in the nucleoplasm and cytoplasm, indicating that there was no defect in mRNA export in these cells (not shown). Thus, it is unlikely that hCRM1 is involved in export of mRNA. Digitonin-permeabilized cells overexpressing the hCRM1-binding domain were severely inhibited in APC-NLS import, comparable to CAN-overexpressing cells and density-arrested U937T cells (not shown). Therefore, a possible additional effect on import of hCRM1 sequestering in the nucleus could not be determined.

Figure 7. Overview of CAN deletion mutants

Black bars represent CAN and CAN mutant proteins, numbers on the left represent amino acid boundaries. Predicted structural motifs are represented as follows: Vertical lines, FG repeats; Diamonds, FXF repeats; LZ, leucine zipper; AH, amphipathic helix. Horizontal stripes indicate an acidic region in the DEK sequence (white bar).

DEK-CAN does not inhibit differentiation of U937 cells

To study the effect of DEK-CAN on myeloid maturation we induced clone DEK-CAN58 to differentiate by using a combination of transforming growth factor β1 (TGFβ1) and 1,25 dihydroxy vitamin D_3 (D3) (Testa et al., 1993) after three days of withdrawal from tetracycline. Surprisingly, the cells died rapidly (Fig. 9A). Immunofluorescence staining showed elevated levels of DEK-CAN in these cells compared to undifferentiated cells (not shown). We consistently found this effect, even when differentiation was induced by other chemicals, such as dimethyl sulfoxide (DMSO) and phorbol 12-myristate 13-acetate (PMA). We, therefore, tried partially releasing the DEK-CAN58 cells for three days in the presence of 5 and 10 ng/ml tetracycline prior to inducing differentiation. Both partially induced and uninduced cells ceased to proliferate upon exposure to TGFβ1 and D3 (Fig. 8A). Compared to uninduced cells (1000 ng/ml tetracycline), viability was not affected in cells cultured with differentiation agents in 10 ng/ml tetracycline, whereas about 50 % of the cells grown in 5 ng/ml tetracycline died. The remaining cells in these cultures expressed high levels of DEK-CAN.



1864-2090	D 1	D 2	D 3	D 4
G0/G1	36	58	55	48
S	50	25	10	9
G2/M	13	15	8	3
Sub-G1	3	2	27	40

Figure 8. The C-terminus of CAN is sufficient to inhibit cell growth

(A) Western blot analysis of 6 % (left panel) and 10 % (right panel) SDS-PAGE gels with lysates from $5x10^5$ induced cells expressing the indicated CAN mutants and DEK-CAN. CAN (1140-1340, 1864-1912, 1984-2090) is abbreviated to CAN 1864-1912+1984-290. Blots were probed as described for Fig. 1A. (B) Growth curves of cells overexpressing selected CAN mutants and DEK-CAN grown in the absence of tetracycline for four days. Data shown are the mean values of triplicate cultures from one experiment of at least three independent experiments that gave similar results. (C) Cell cycle phase distribution of induced CAN 1864-2090 cells. (D) DNA content was quantitated by flow cytometric analysis in CAN 1864-2090 cells, overexpressing the hCRM1-binding domain, at day 1, 2, 3, and 4 after withdrawal of tetracycline.

DEK-CAN58 cells grown in 5 and 1000 ng/ml tetracycline were induced to differentiate by a 5-day exposure to TGFβ1 and D3. As a measure for induction of differentiation, we monitored the increased expression of the cell surface antigens CD11a, CD11b, CD14, and CD18. The enhanced expression of these markers indicated that DEK-CAN-expressing DEK-CAN58 cells (Fig. 8B, right panel) differentiated normally to mature monocytes in a manner indistinguishable from tetracycline-repressed DEK-CAN58 cells (Fig. 8B, left panel). Moreover, Giemsa-staining of the two differentiated cell populations showed the same morphology (not shown), confirming that DEK-CAN expression did not inhibit the myeloid differentiation of U937 cells.

Discussion

We examined the biologic properties of the CAN protein and the leukemia-specific DEK-CAN fusion protein by overexpressing these proteins in U937 myeloid precursor cells. We found that ectopic expression of CAN caused a cell cycle arrest in G_0, followed by a block in mRNA export and apoptotic cell death. Overexpression of the most C-terminal FG-repeat-containing region of CAN, which binds hCRM1, was sufficient to reproduce most of these effects: it induced growth arrest and apoptosis. Thus, overexpression of CAN and the hCRM1-binding domain of CAN interfered with some of CAN's normal functions in a dominant-negative way. Moderate DEK-CAN expression slightly inhibited cell growth and did not interfere with the differentiation of U937 cells to mature monocytes. Because U937 cells represent an intermediate stage of monocytic development (Hilfinger et al., 1993), it is possible that their terminal differentiation is not affected because DEK-CAN may only inhibit differentiation of earlier myeloid precursors. Myeloid cells from patients with t(6;9) acute myeloid leukemia are partially inhibited in their differentiation pathways but are not totally blocked. Therefore, it is also possible that DEK-CAN has no effect on differentiation but affects proliferation of early precursor cells in t(6;9) patients.

CAN overexpression induces cell cycle arrest and apoptosis

CAN-overexpressing cells, and to a lesser extent cells overexpressing the hCRM1-interaction domain of CAN, accumulated with a diploid DNA content. Arrested CAN-overexpressing cells no longer express *c-myc*, a proto-oncogene that is continuously expressed in proliferating cells but is downregulated when cells exit the cell cycle (Freytag, 1988; Waters et al., 1991). U937T cells that were mostly arrested in G_0/G_1 after reaching their maximal density, did not import detectable levels of import substrate. Therefore, additional effects of the overexpression of CAN and the hCRM1-binding domain on nuclear protein import could not be measured. It is possible that CAN overexpression interferes with the nuclear transport of factors critical to cell growth or survival. Alternatively, proper stoichiometry of the components that make up the NPC could be necessary for the formation of new NPCs, a process that is presumably essential for growth. CAN depletion leads to cell cycle arrest in G_2 (van Deursen et al., 1996), whereas overexpression of CAN arrested cells in G_0, suggesting that CAN is essential to proper cell cycle progression. Furthermore, CAN-depleted cells still have hCRM1 in their nuclear envelope (Fornerod et al., 1997), whereas the nuclear envelope of CAN-overexpressing cells is depleted of hCRM1 (see below). It will be interesting to see how the transport function of hCRM1 could be linked to cell cycle progression.

Cells overexpressing full-length CAN or CAN mutants containing the hCRM1-binding domain die after 72 to 96 hr of induction, and show morphologic features of apoptosis. Co-expression of the potent survival factor Bcl-X_L did not protect CAN-overexpressing cells from apoptosis, suggesting that a non-Bcl-X_L-controlled pathway is activated.

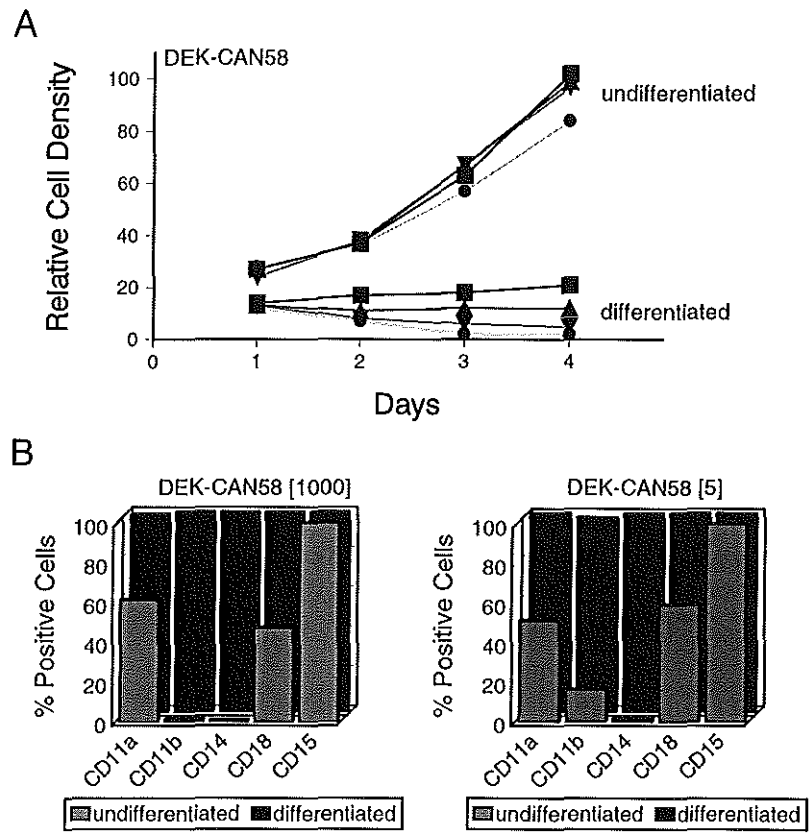


Figure 9. Differentiation antigen expression in U937T cells expressing DEK-CAN

(A) Growth curves of DEK-CAN58 cells cultured in medium containing 1000 (■), 10 (▲), 5 (▼) or 0 (●) ng/ml tetracycline, seeded at $1x10^5$ cells/ml in the presence (differentiated) or absence (undifferentiated) of 1 ng/ml TGFβ1 and 250 ng/ml D3. The cells were cultured in normal medium containing the indicated tetracycline concentrations for three days prior to the induction of differentiation. (B) DEK-CAN58 cells cultured in 1000 ng/ml tetracycline (no DEK-CAN expression) and 5 ng/ml tetracycline (partial DEK-CAN induction) were differentiated in medium containing TGFβ1 and D3 for five days (differentiated; black bars). Undifferentiated cells cultured in 1000 and 5 ng/ml tetracycline are also shown (undifferentiated; gray bars). Expression of the indicated differentation antigens (CD11a, CD11b, CD14, CD15, CD18) was evaluated by cytofluorimetry using specific monoclonal antibodies. Results are expressed as the percentage of antigen-positive cells.

hCRM1 function is essential

hCRM1, recently identified as a CAN-associating protein, is a putative transport factor that normally localizes to the nucleus and the NPC, and is regularly released from the NPC into the nucleoplasm (Fornerod et al., 1997). CAN mutants containing the hCRM1-binding region were, at least in part, nuclear and caused nuclear accumulation of hCRM1. Overexpression of these mutants at levels that were lethal, resulted in a complete depletion of hCRM1 from the nuclear envelope. This result suggests that the excess of nuclear hCRM1-binding domain competes with NPC-associated CAN for binding to hCRM1 and inhibits a function of hCRM1 that is essential for cell growth or survival.

The localization of overexpressed full-length CAN changed from mainly nuclear membrane and cytoplasm after the first day of induction, to mainly nuclear during the second and third day of expression. HeLa cells, transiently transfected to highly overexpress CAN, showed a similar nuclear localization in 5-10 % of the cells (Fornerod et al., 1995). It may be that overexpressed CAN spills over into the cytoplasm and nucleus when all of the NPC binding sites are saturated. Transport of CAN into the nucleus could be mediated by its association with hCRM1 (Fornerod et al., 1996). The colocalization of CAN and hCRM1 in cytoplasmic structures suggests that complex formation already occurs in the cytoplasm. Since hCRM1 has a half-life of approximately 24 hours (Fornerod et al., 1997), it is unlikely that the hCRM1 observed in the cytoplasm of CAN-overexpressing cells is only newly synthesized hCRM1. Instead, these data suggest that hCRM1 travels from the nucleus to the cytoplasm, in addition to its release from the NPC into the nucleoplasm. In normal HeLa cells, only a small amount of hCRM1 is present in cytoplasmic annulate lamellea (Fornerod et al., 1997).

The region of CAN required for its colocalization with hCRM1 in the nucleus was smaller than the hCRM1-binding domain identified by coimmunoprecipitation (Fornerod et al., 1996), suggesting that the *in vivo* interaction of mutants that lack part of the binding domain is too weak to be detected by immunoprecipitation. This finding is consistent with the idea that nuclear localization of the C-terminal repeat region of CAN, which does not contain a known NLS, is mediated by hCRM1 (Fornerod et al., 1996). As yet, we do not have clues regarding the transport substrate of hCRM1. All we can say with certainty is that it is not essential for mRNA export. The hCRM1-binding domain overexpressing U937 cells should provide a good model to investigate potential substrates.

CAN overexpression induces defects in p97 localization and mRNA export

Overexpressed CAN colocalized with the nuclear import factor p97/importin β, initially in the nuclear membrane and cytoplasmic structures, and subsequently in nuclear structures. p97 binds to CAN *in vitro* (Radu et al., 1995a), but this interaction is not strong enough to mediate co-immunoprecipitation with CAN from cell lysates of CAN-overexpressing cells (Fornerod et al., 1997). Based on binding studies to NUP98 mutants and other nucleoporins, the FG-repeat-regions are thought to harbor the p97-binding domain (Moroianu et al., 1995b; Radu et al., 1995b). Our results with CAN 1558-1840 and CAN 1864-2090 show that p97 does not bind to the overexpressed C-terminal CAN repeat regions alone. Instead, p97 colocalizes with full-length CAN and partly with CAN 817-2090, both of which bind to the NPC and form structures of unknown composition upon overexpression. It is possible that p97 associates *in vivo* also with the central region of CAN, either directly or via another protein, and that binding of p97 to CAN requires both additive interactions.

Only cells overexpressing full-length CAN demonstrated a defect in polyadenylated RNA export after 55-60 hr of induction. These were also the only cells to sequester p97 in their nuclei. These observations may be directly linked because depletion of p97 from the cytoplasm may lead to a block in importin α import into the nucleus, thereby inhibiting its function in the export of capped RNAs (Görlich et al., 1996). Studies in yeast cells overexpressing the nucleoporin gene *NUP116* also suggest that p97 plays a role in mRNA export. Overexpression of the GLFG-repeat region of Nup116p severely inhibits cell growth and blocks polyadenylated RNA export (Iovine et al., 1995). This region interacts with Kap95p, an essential yeast homolog of the vertebrate import factor p97, suggesting that sequestering of this factor is at least partly responsible for the phenotype (Iovine et al., 1995).

In summary, our results show that the cytotoxicity of CAN overexpression is caused by depletion of hCRM1 from the nuclear envelope, thereby inhibiting an essential function of hCRM1. Furthermore, only full-length CAN, which contains the central protein-protein interaction domain in addition to the C-terminal FG-repeats colocalizes with p97 in the nucleus and causes nuclear accumulation of polyadenylated RNA.

The mechanism by which DEK-CAN contributes to leukemogenesis remains unknown. DEK is a sequence-specific DNA-binding protein, and DEK binding sites were recently identified in the regulatory regions of several early myeloid genes (Fu et al., 1997). DEK-CAN could exhibit altered transcriptional regulation compared with DEK, due to the presence of CAN sequences or proteins that associate with this portion of CAN, such as hCRM1. Alternatively, the redistibution of hCRM1 towards the nucleoplasm by DEK-CAN could interfere with the transport function of CAN and hCRM1. DEK-CAN is expressed at such a low level in leukemic cells, that a total

depletion of hCRM1 from the NPC is not to be expected, but a shift in the balance of nuclear hCRM1 may have an effect on hematopoietic cell growth or differentiation.

Materials and Methods

Expression constructs

All expression plasmids used in this study carry sequences encoding two copies of the influenza virus hemagglutinin (HA1) tag at the 5' end of their open reading frames (Fornerod et al., 1995) and are driven by the tetVP16-responsive promoter (Gossen and Bujard, 1992). Expression of CAN, DEK-CAN, CAN 1-1058, CAN 586-1058, CAN 816-2090, CAN 1864-2090, and CAN 1558-1840 was directed by plasmids described previously (Fornerod et al., 1995; Fornerod et al., 1996). CAN 1140-2090 expression was directed by plasmid pHA1-Can Δ1-1139 (Fornerod et al., 1995). Construct pHA1-CAN (1140-1340, 1864-1912, 1984-2090) was derived from pHA1-CAN (1140-1340, 1864-2090) by an in-frame deletion using the BamHI sites at positions 5825 and 6044 of the *can* cDNA. Plasmid pHA1-CAN 1864-2017 was derived by subcloning the region that encodes amino acids 1864-2090 from pHA1-CAN (Δ1-1139, Δ1341-1863) and inserting an oligonucleotide containing translational stops in the PstI site at position 6145 of the *can* cDNA. A plasmid containing the murine Bcl-X_L cDNA driven by the SFFV LTR was kindly provided by Dr G. Nuñez.

Inducible gene expression in U937T cells

The human monoblast cell line U937 (Sundström and Nilsson, 1976) and its derivatives were routinely cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum in a 37°C incubator with 5 % CO_2. Transfections were performed by electroporation at 0.17 kV, 960 µF on a Biorad Gene pulser. To generate cell lines inducibly expressing the proteins of interest, we used a two-step procedure. First, U937 cells were transfected with pUHD/*TetVP16*Puro (Vignali et al., 1996), encoding tetVP16 under the control of the tetVP16-responsive promoter (Gossen and Bujard, 1992), thereby making tetVP16 expression tetracycline-repressible and autoregulatory (Shockett et al., 1995). Transfected cells were selected in 0.5 µg/ml puromycin in the presence of 1 µg/ml tetracycline. Single clones were examined for tetVP16 expression by RNA dot blot of cells grown in the presence or absence of tetracycline. Of the 18 clones examined seven showed tetracycline-controlled expression of tetVP16. Second, clone U937T was selected for subsequent stable transfection with expression constructs containing HA1-epitope tagged (tt) cDNAs under control of the tetVP16-responsive promoter. pHA1-CAN was cotransfected with the neomycin-selectable pMC1NeoPolyA plasmid (Stratagene), whereas all other expression plasmids were cotransfected with the hygromycin-selectable pGEMHyg plasmid (van Deursen et al., 1991). Clones were selected and maintained in the presence of 1 µg/ml tetracycline. Independent single clones were examined for tetracycline-dependent expression by Western blot analysis (Sambrook et al., 1989) using the anti-HA1 epitope monoclonal antibody 12CA5 (Boehringer) at 2 µg/ml. Bound antibody was visualized with a peroxidase-conjugated goat anti-mouse antibody (Jackson) and chemiluminescence reagent (NEN) used according to the manufacturer's instructions.

Cell growth, DNA content analysis, and apoptosis

For induction of tetracycline-controlled gene expression, cells were washed four times with 10 ml PBS and seeded at 10^5 cells/ml in complete medium containing the desired tetracycline concentration. Cell proliferation was measured using a non-radioactive cell proliferation assay (Promega) according to the manufacturer's instructions. DNA content and cell cycle distribution were evaluated by flow cytometric analysis of propidium iodide-stained nuclei as described previously (Nicoletti et al., 1991). Fragmented

DNA was isolated as previously described (Martin et al., 1990) and separated on 1 % agarose gels containing ethidium bromide.

Differentiation of U937 cells
Induced DEK-CAN58 cells were cultured for five days in the presence of 1 ng/ml TGFβ1 (Promega) and 250 ng/ml D3 (Biomol). The percentages of monocyte surface antigen-positive cells were evaluated by FACS analysis five days after induction of differentiation. The following mouse monoclonal antibodies were used: anti-CD11a, anti-CD11b (MO1), anti-CD14 (MY4), anti-CD15, and anti-CD18.

Indirect immunofluorescence
Cytospins of U937 cells were fixed, permeabilized, and immunostained as described (Fornerod et al., 1995). Primary antibodies were diluted 1 in 400 for αCNC (Fornerod et al., 1995), 2 µg/ml for 12CA5, 2 µg/ml for mAb3E9 (Chi et al., 1995), and 1 in 90 for affinity-purified α-hCRM1 (Fornerod et al., 1997). Bound primary antibodies were visualized with goat secondary antibodies conjugated to FITC (Sigma) or Texas Red (USB). Images were obtained by confocal laser scanning microscopy on a BioRad MRC1000 using a 60x oil objective.

Transport assays
In vitro nuclear protein import assays were performed essentially as described (Adam et al., 1990), with MDBK lysate instead of reticulocyte lysate as a source of essential cytosolic factors. Import was allowed to proceed for 20 min at 27°C, with parallel reactions on ice as controls. Immediately following import, cells were washed and fixed in 3 % formaldehyde for 15 min on ice. The mean fluorescence of the accumulated APC-NLS substrate in these cells was determined by FACS analysis. To determine the intracellular distribution of polyadenylated RNA, we hybridized cells on sterile cytospins with an oligo$(dT)_{50}$ probe directly coupled to FITC as described (van Deursen et al., 1996). Images were obtained by confocal laser scanning microscopy on a BioRad MRC1000 using a 60x oil objective.

Electron Microscopy
CAN7 cells were induced for 60 hr and then fixed for 2 hr in 0.1 M phosphate buffer, pH 7.4, containing 2 % glutaraldehyde. Cells were then post-fixed in 1 % osmium tetroxide for 1 hr, dehydrated, and embedded in Spurr low-viscosity resin (EMS). Thin sections were cut, stained with uranyl acetate and lead, and examined with a JEOL JEM-1200EX II electron microscope.

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References

Adam, E. J. H., and Adam, S. A. (1994). Identification of cytosolic factors required for nuclear location sequence-mediated binding to the nuclear envelope. J Cell Biol *125*, 547-555.

Adam, S. A., and Gerace, L. (1991). Cytosolic proteins that specifically bind nuclear localization signals are receptors for nuclear import. Cell *66*, 837-847.

Adam, S. A., Marr, R. S., and Gerace, L. (1990). Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. J Cell Biol *111*, 807-816.

Amberg, D. C., Goldstein, A. L., and Cole, C. N. (1992). Isolation and characterization of *RAT1*: An essential gene of *Saccharomyces cerevisiae* required for the efficient nucleocytoplasmic trafficking of mRNA. Genes & Dev *6*, 1173-1189.

Boise, L. H., González-García, M., Postema, C. E., Ding, L., Lindsten, T., Turka, L. A., Mao, X., Nuñez, G., and Thompson, C. B. (1993). bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell *74*, 597-608.

Borner, C. (1996). Diminished cell proliferation associated with the death-protective activity of Bcl-2. J Biol Chem *271*, 12695-12698.

Chi, N. C., Adam, E. J. H., and Adam, S. A. (1995). Sequence and characterization of cytoplasmic nuclear protein import factor p97. J Cell Biol *130*, 265-274.

Davis, L. I. (1995). The nuclear pore complex. Annu Rev Biochem *64*, 865-896.

Davis, L. I., and Fink, G. R. (1990). The NUP1 gene encodes an essential component of the yeast nuclear pore complex. Cell *61*, 965-978.

Doye, V., and Hurt, E. C. (1995). Genetic approaches to nuclear pore structure and function. Trends Genet *11*, 235-241.

Fabre, E., and Hurt, E. C. (1994). Nuclear transport. Curr Opin Cell Biol *6*, 335-342.

Fornerod, M., Boer, J., van Baal, S., Jaeglé, M., Von Lindern, M., Murti, K. G., Davis, D., Bonten, J., Buijs, A., and Grosveld, G. (1995). Relocation of the carboxyterminal part of CAN from the nuclear envelope to the nucleus as a result of leukemia-specific chromosome rearrangements. Oncogene *10*, 1739-1748.

Fornerod, M., Boer, J., van Baal, S., Morreau, H., and Grosveld, G. (1996). Interaction of cellular proteins with the leukemia specific fusion proteins DEK-CAN and SET-CAN and their normal counterpart, the nucleoporin CAN. Oncogene *13*, 1801-1808.

Fornerod, M., van Deursen, J., van Baal, S., Reynolds, A., Davis, D., Murti, K. G., and Grosveld, G. (1997). The human homologue of yeast CRM1 is in a dynamic subcomplex with CAN/Nup214 and a novel nuclear pore component Nup88. EMBO J *16*, 807-816.

Freytag, S. O. (1988). Enforced expression of the c-*myc* oncogene inhibits cell differentiation by precluding entry into a distinct predifferentiation state in G0/G1. Mol Cell Biol *8*, 1614-1624.

Fu, G., Grosveld, G., and Markovitz, D. (1997). DEK, an autoantigen involved in a chromosomal translocation in acute myelogenous leukemia, binds to the HIV-2 enhancer. Proc Natl Acad Sci USA *94*, 1811-1815.

Gavrieli, Y., Sherman, Y., and Ben-Sasson, S. A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol *119*, 493-501.

Gerace, L. (1995). Nuclear export signals and the fast track to the cytoplasm. Cell *82*, 341-344.

González-García, M., Garcia, I., Ding, L., O'Shea, S., Boise, L. H., Thompson, C. B., and Nuñez, G. (1995). bcl-x is expressed in embryonic and postnatal neural tissues and functions to prevent neuronal cell death. Proc Natl Acad Sci USA *92*, 4304-4308.

Görlich, D., Kraft, R., Kostka, S., Vogel, F., Hartmann, E., Laskey, R. A., Mattaj, I. W., and Izaurralde, E. (1996). Importin provides a link between nuclear protein import and U snRNA export. Cell *87*, 21-32.

Görlich, D., and Mattaj, I. (1996). Nucleocytoplasmic transport. Science *271*, 1513-1518.

Görlich, D., Prehn, S., Laskey, R. A., and Hartmann, E. (1994). Isolation of a protein that is essential for the first step of nuclear protein import. Cell *79*, 767-778.

Görlich, D., Vogel, F., Mills, A. D., Hartmann, E., and Laskey, R. A. (1995). Distinct functions for the two importin subunits in nuclear protein import. Nature *377*, 246-248.

Gossen, M., and Bujard, H. (1992). Tight control of gene expression in mammelian cells by tetracyclin-responsive promoters. Proc Natl Acad Sci USA *89*, 5547-5551.

Gottschalk, A. R., Boise, L. H., Oltvai, Z. N., Accavitti, M. A., Korsmeyer, S. J., Quintáns, J., and Thompson, C. B. (1996). The ability of bcl-x_L and bcl-2 to prevent apoptosis can be differentially regulated. Cell Death Diff *3*, 113-118.

Hilfinger, J. M., Clark, N., Smith, M., Robinson, K., and Markovitz, D. M. (1993). Differential regulation of the human immunodeficiency virus type 2 enhancer in monocytes at various stages of differentiation. J Virol *67*, 4448-53.

Iovine, M. K., Watkins, J. L., and Wente, S. R. (1995). The GLFG repetitive region of the nucleoporin Nup116p interacts with Kap95p, an essential yeast nuclear import factor. J Cell Biol *131*, 1699-1713.

Izaurralde, E., and Mattaj, I. W. (1995). RNA export. Cell *81*, 153-159.

Kelly, K., and Siebenlist, U. (1986). The regulation and expression of c-myc in normal and malignant cells. Annu Rev Immunol *4*, 317-338.

Kraemer, D., Wozniak, R. W., Blobel, G., and Radu, A. (1994). The human can protein, a putative oncogene product associated with myeloid leukemogenesis, is a nuclear pore complex protein that faces the cytoplasm. Proc Natl Acad Sci USA *91*, 1519-1523.

Kraemer, D. M., Strambio-de-Castillia, C., Blobel, G., and Rout, M. P. (1995). The essential yeast nucleoporin NUP159 is located on the cytoplasmic side of the nuclear pore complex and serves in karyopherin-mediated binding of transport substrate. J Biol Chem *270*, 19017-19021.

Martin, S. J., Lennon, S. V., Bonham, A. M., and Cotter, T. G. (1990). Induction of apoptosis (programmed cell death) in human leukemic HL-60 cells by inhibition of RNA or protein synthesis. J Immunol *145*, 1859-67.

Melchior, F., Paschal, B., Evans, J., and Gerace, L. (1993). Inhibition of nuclear protein import by nonhydrolyzable analogues of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor [published erratum appears in J Cell Biol 124:217, 1994]. J Cell Biol *123*, 1649-1659.

Moore, M. S., and Blobel, G. (1993). The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. Nature *365*, 661-663.

Moroianu, J., Blobel, G., and Radu, A. (1995a). Previously identified protein of uncertain function is karyopherin α and together with karyopherin β docks import substrate at nuclear pore complexes. Proc Natl Acad Sci USA *92*, 2008-2011.

Moroianu, J., Hijikata, M., Blobel, G., and Radu, A. (1995b). Mammalian karyopherin α1β and α2β heterodimers: α1 or α2 subunit binds nuclear localization signal and β subunit interacts with peptide repeat-containing nucleoporins. Proc Natl Acad Sci USA *92*, 6532-6536.

Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F., and Riccardi, C. (1991). A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Meth *139*, 271-279.

Panté, N., and Aebi, U. (1994). Toward the molecular details of the nuclear pore complex. J Struct Biol *113*, 179-189.

Radu, A., Blobel, G., and Moore, M. S. (1995a). Identification of a protein complex that is required for nuclear protein import and mediates docking of import substrate to distinct nucleoporins. Proc Natl Acad Sci USA *92*, 1769-1773.

Radu, A., Moore, M. S., and Blobel, G. (1995b). The peptide repeat domain of nucleoporin Nup98

functions as a docking site in transport across the nuclear pore complex. Cell *81*, 215-222.

Rexach, M., and Blobel, G. (1995). Protein import into nuclei: Association and dissociation reactions involving transport substrate, transport factors, and nucleoporins. Cell *83*, 683-692.

Rout, M. P., and Wente, S. R. (1994). Pores for thought: Nuclear pore complex proteins. Trends Biochem Sci *4*, 357-363.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, J. Sambrook, E. F. Fritsch and T. Maniatis, eds. (Cold Spring Harbor Laboratory: Cold Spring Harbor, New York).

Sherr, C. J. (1996). Cancer cell cycles. Science *274*, 1672-1677.

Shockett, P., Difilipantonio, M., Hellman, N., and Schatz, D. (1995). A modified tetracyclin regulated system provides autoregulatory inducible gene expression in cultured cells and transgenic mice. Proc Natl Acad Sci USA *92*, 6522-6526.

Sundström, C., and Nilsson, K. (1976). Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int J Cancer *171*, 565-577.

Testa, U., Masciulli, R., Tritarelli, E., Pustorino, R., Mariani, G., Martucci, R., Barberi, T., Camagna, A., Valtieri, M., and Peschle, C. (1993). Transforming growth factor-beta potentiates vitamin D3-induced terminal monocytic differentiation of human leukemic cell lines. J Immunol *150*, 2418-30.

van Deursen, J., Boer, J., Kasper, L., and Grosveld, G. (1996). G_2 arrest and impaired nucleocytoplasmic transport in mouse embryos lacking the proto-oncogene *CAN/Nup214*. EMBO J *15*, 5574-5583.

van Deursen, J., Lovell-Badge, R., Oerlemans, F., Schepens, J., and Wieringa, B. (1991). Modulation of gene activity by consecutive gene targeting of one creatine kinase M allele in mouse embryonic stem cells. Nucl Acids Res *19*, 2637-2643.

Vignali, D. A., Carson, R. T., Chang, B., Mittler, R. S., and Strominger, J. L. (1996). The two membrane proximal domains of CD4 interact with the T cell receptor. J Exp Med *183*, 2097-2107.

Von Lindern, M., Fornerod, M., van Baal, S., Jaeglé, M., de Wit, T., Buijs, A., and Grosveld, G. (1992). The translocation (6;9), associated with a specific subtype of acute myeloid leukemia, results in the fusion of two genes, dek and can, and the expression of a chimeric, leukemia-specific dek-can mRNA. Mol Cell Biol *12*, 1687-1697.

Waters, C., Littlewood, T., Hancock, D., Moore, J., and Evan, G. (1991). c-*myc* protein expression in untransformed fibroblasts. Oncogene *6*, 101-109.

Weis, K., Mattaj, I. W., and Lamond, A. I. (1995). Identification of hSRP1 alpha as a functional receptor for nuclear localization sequences. Science *268*, 1049-1053.

Wente, S. R., and Blobel, G. (1993). A temperature-sensative NUP116 null mutant forms a nuclear envelope seal over the yeast nuclear pore complex thereby blocking nucleocytoplasmic traffic. J Cell Biol *123*, 275-284.

Wozniak, R. W., Blobel, G., and Rout, M. P. (1994). POM152 is an integral protein of the pore membrane domain of the yeast nuclear envelope. J Cell Biol *125*, 31-42.

4 The nucleoporin CAN/Nup214 binds to both the cytoplasmic and nucleoplasmic sides of the nuclear pore complex in overexpressing cells

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Abstract

CAN/Nup214, an essential component of the vertebrate nuclear pore complex (NPC), is required for proper cell cycle progression and nucleocytoplasmic transport. It is a member of the FG-repeat-containing family of nucleoporins and has been localized to the cytoplasmic face of the NPC. Indirect immunofluorescence studies with specific antibodies have shown that moderate overexpression of human *CAN* in HeLa cells causes an increase in CAN/Nup214 levels at the nuclear envelope. Here, we demonstrate that in such HeLa cells, CAN/Nup214 does not localize exclusively to the cytoplasmic side of the NPC. Cryosections, stained with CAN-specific antibodies and examined by electron microscopy, showed that about one-third of the gold-labeled NPCs were decorated at the cytoplasmic face and the remaining two-thirds at the nucleoplasmic face. These data indicate that both the cytoplasmic fibrils and the nuclear basket of the vertebrate NPC contain specific binding sites for either CAN/Nup214 or for its interacting proteins, Nup88 and hCRM1. Thus, it is conceivable that CAN/Nup214 functions in nucleocytoplasmic transport at both faces of the NPC.

Introduction

The vertebrate nuclear pore complex (NPC), a ~125 MDa multiprotein assembly that mediates bidirectional transport between the cytoplasm and the nucleus, is composed of a spoke complex embracing a central channel or transporter. A cytoplasmic ring with filaments emanating into the cytoplasm is attached to the spoke complex, as is a nuclear ring with filaments that converge to form the nuclear basket (for reviews see Davis, 1995; Panté and Aebi, 1993). The NPC consists of multiple copies of an estimated 100 different proteins (nucleoporins), including integral membrane proteins and peripheral proteins. A number of these nucleoporins contain multiple degenerate peptide motifs, that share an FG dipeptide core sequence (reviewed by Davis, 1995; Fabre and Hurt, 1994; Panté and Aebi, 1994; Rout and Wente, 1994).

Protein import into the nucleus is mediated by a heterodimer comprising the nuclear localization signal (NLS)-receptor/importin α, which recognizes the NLSs of karyophilic proteins, and nuclear import factor p97/importin β, which contacts the NPC (reviewed by Görlich and Mattaj, 1996; Melchior and Gerace, 1995; Panté and Aebi, 1996; Schlenstedt, 1996). After this first docking step, the ligand complex is imported via an energy-dependent process that requires the small GTPase Ran/TC4 (Melchior et al., 1993; Moore and Blobel, 1993). GTP hydrolysis by Ran is thought to drive the translocation reaction (Corbett et al., 1995; Melchior et al., 1995; Schlenstedt et al., 1995), which may involve repeated association and dissociation of import complex components to the NPC (Nehrbass and Blobel, 1996; Rexach and Blobel, 1995), or the movement of a single complex across the NPC either in discrete steps (Görlich et al., 1996a), or in a sliding motion (Schlenstedt, 1996). Many FG-repeat-containing nucleoporins are located at distinct positions along the NPC and may form binding sites for transport complexes (Radu et al., 1995a; Radu et al., 1995b; Rexach and Blobel, 1995). Protein and ribonucleoprotein (RNP) export from the nucleus is also a receptor-mediated, energy-dependent process (for reviews see Görlich and Mattaj, 1996; Izaurralde and Mattaj, 1995), and involves some of the same factors necessary for protein import (Görlich et al., 1996).

The FG-repeat-containing nucleoporin CAN, also called Nup214 and p250, is detected exclusively at the cytoplasmic face of the NPC in HeLa cells (Kraemer et al., 1994). In *Xenopus* oocytes CAN was sublocalized to the cytoplasmic filaments of the NPC (Panté et al., 1994). Recently, we made a knockout mutation of the mouse *CAN* gene. Mouse embryos that lack CAN show growth arrest, followed by simultaneous defects in polyadenylated RNA export and protein import (van Deursen et al., 1996). Further, coimmunoprecipitation experiments demonstrated that CAN is part of a complex with at least two other polypeptides - one of 88 kDa, and the other of 112 kDa (Fornerod et al., 1996). Recently, these proteins were identified as the novel NPC

component Nup88 and hCRM1, a homolog of yeast CRM1 that is localized to the NPC and nucleoplasm (Fornerod et al., 1997).

In previous studies, we found that overexpression of CAN in HeLa-derived cells results in an increase of this nucleoporin at the nuclear envelope (Fornerod et al., 1995). To examine the sublocalization of overexpressed CAN within the NPC, we have used ultrathin cryosections in combination with immunogold labeling techniques.

Results

CE490S cells can be induced to overexpress CAN because they contain the *CAN* cDNA, under the control of the tet-operator promoter (Fornerod et al., 1995; Gossen and Bujard, 1992). In the presence of 1 µg/ml tetracycline, CE490S cells express endogenous levels of CAN. However, if tetracycline is removed from the medium, CAN is moderately overexpressed, without detectably affecting cell growth or nuclear envelope structure. Indirect immunofluorescence studies using our polyclonal αCNN antiserum, which is directed against unique sequences in the N-terminus of CAN,

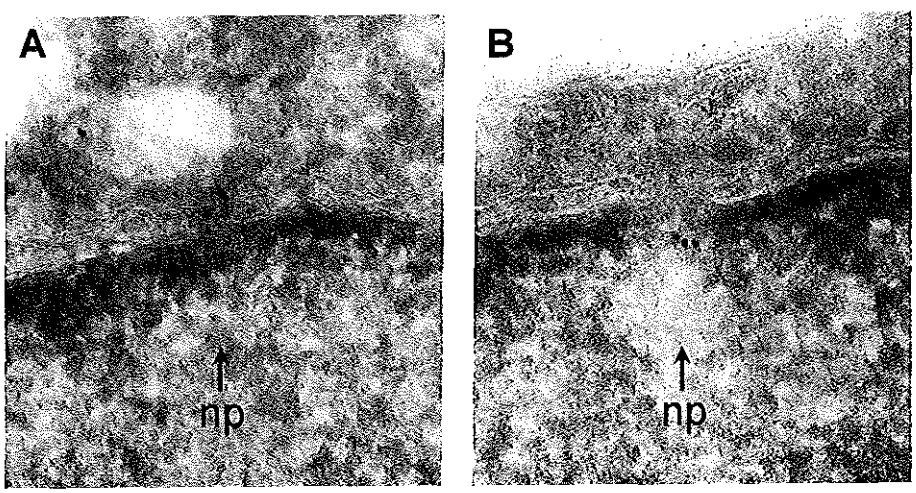

Figure 1. CAN localizes to both sides of the NPCs of CAN-overexpressing CE490S cells

Ultrathin cryosections were immunolabeled with polyclonal αCNN antiserum, followed by 10 nm gold-conjugated protein A. Selected examples of labeled NPC cross-sections are aligned with their cytoplasmic and nucleoplasmic sides up and down, respectively. The NPCs are indicated (np). (A) Localization of epitopes of N-terminal CAN at both the cytoplasmic and nucleoplasmic sides of the NPC. (B) Gold decorating the nucleoplasmic side of the NPC. Magnification is 120,000 times.

showed that the protein is located mainly at the nuclear envelope in cells expressing normal CAN levels and in cells overexpressing CAN (Fornerod et al., 1995).

For ultrastructural localization studies, ultrathin cryosections of CE490S cells were first incubated with αCNN, followed by protein A complexed to 10 nm gold particles. In CAN overexpressing cells, gold particles decorated both cytoplasmic and nucleoplasmic sides of the NPCs (Fig.1). No significant label was found in the cytoplasm or nucleoplasm, indicating that the overexpressed protein specifically associated with the NPCs. Quantitation of the gold distribution in 100 nuclei showed that 59% of the NPCs were decorated at the nucleoplasmic side (Fig.1B), 36% at the cytoplasmic side (data not shown), and 5% at both sides or in the middle (Fig.1A). The amount of label detected at both sides of the NPCs varied, which is probably due to the plane of section. Our antibody did not detect gold label in CE490S cells expressing endogenous levels of CAN (data not shown).

Discussion

Our immunoelectron microscopic studies on CAN-overexpressing HeLa cells showed that CAN localized to both the cytoplasmic and nucleoplasmic sides of the NPC. This result indicates that specific binding sites for CAN, or for its interacting proteins, exist on both faces of the nuclear pore. The NPCs of normal HeLa cells did not stain, which demonstrates that immunoelectron detection of CAN with our antiserum, directed against N-terminal CAN sequences, requires elevated levels of the protein. In previous immunolocalization studies CAN is only detected at the cytoplasmic face of NPCs of normal HeLa cells and *Xenopus* oocytes (Kraemer et al., 1994; Panté et al., 1994). Why is CAN found at the nucleoplasmic side of the NPC in overexpressing HeLa cells?

Studies on normal HeLa cells employed a specific antibody against C-terminal CAN sequences, that localized CAN to the cytoplasmic face of NPCs (Kraemer et al., 1994). It is conceivable that epitopes in the C-terminal part of CAN are masked specifically at the nucleoplasmic side of the NPC. This region may for instance interact with certain proteins or protein complexes in the nucleus, thereby preventing antibody binding. On the other hand, the levels of endogenous CAN at the nucleoplasmic side may be too low to detect with the C-terminal CAN antiserum.

CAN has also been detected at the cytoplasmic fibrils of *Xenopus* oocyte NPCs with antibodies directed against the entire protein (Panté et al., 1994). Interestingly, the FG-repeat-containing nucleoporin p62 is found exclusively at the nucleoplasmic face of NPCs of *Xenopus* oocyte nuclear envelopes (Cordes et al., 1991; Panté and Aebi, 1993); however, in mammalian liver cells, this protein localizes to both sides of the NPC (Cordes et al., 1991; Guan et al., 1995). Consequently, the sublocalization of

nucleoporins at the NPC may vary with cell-type or with the expression level of the nucleoporin.

Altered expression levels of nucleoporins may potentially disrupt the normal distribution or architecture of the NPCs. However, our electron micrographs showed no gross alterations of nuclear envelope structure, such as herniations or NPC clustering (see, e.g., Wente and Blobel, 1994; Wente and Blobel, 1993). Moreover, the growth rate of CE490S cells was not affected, suggesting that moderately elevated CAN levels do not disrupt nucleocytoplasmic transport (Iovine et al., 1995). Finally, it cannot be absolutely excluded that even mild overexpression of CAN induces an artificial localization of the protein at the nuclear side of the NPC, without any relevance to the mechanism of nucleocytoplasmic trafficking. However, the specificity of the localization of overexpressed CAN protein is striking: CAN is exclusively detected at the NPCs, and not in any other cellular compartment or structure. This argues that CAN has the ability to bind specifically to nucleoplasmic components of the nuclear pore complex, in addition to its established association with the cytoplasmic fibrils, and that mildly overexpressing cells could very well reinforce a normal biological situation.

Repeat-containing NPC proteins, such as CAN, are believed to play an important role in transport across the NPC, but the precise function of the repeats in this process is unknown (Fabre and Hurt, 1994; Rout and Wente, 1994). To date, *in vitro* studies have shown that FG peptide motifs may serve as binding sites along the pore for specific factors carrying transport substrates (Iovine et al., 1995; Moroianu et al., 1995; Radu et al., 1995a; Radu et al., 1995b). Each NPC protein that has been localized to a specific subregion of the NPC, with the exception of p62, is confined to a specific niche either on the cytoplasmic or the nucleoplasmic side. Immunoelectron microscopy has demonstrated that RanBP2/Nup358 localizes to the cytoplasmic fibrils (Kraemer et al., 1994; Panté et al., 1994; Wilken et al., 1995; Wu et al., 1995; Yokoyama et al., 1995), whereas Nup153 and Nup98 are constituents of the nuclear basket (Cordes et al., 1993; Panté et al., 1994; Radu et al., 1995b; Sukegawa and Blobel, 1993). These findings have provided the basis for several transport models in which FG-repeat-containing nucleoporins form a static array of docking sites that transiently interact with transport complexes (Görlich and Mattaj, 1996; Radu et al., 1995b; Schlenstedt, 1996).

Based on our subcellular localization data it appears that CAN could interact with transport complexes at both sides of the NPC. CAN depletion in mouse embryos results in defects in both nuclear protein import and mRNA export. Therefore, a symmetric distribution of CAN may be important for bidirectional transport of different substrates through the nuclear pore. Like CAN, other nucleoporins could also localize to multiple sites within the NPC. In this respect, it is interesting that Cordes et al (1993) found a small but significant amount of Nup153 at the cytoplasmic side of the NPC of mouse liver cells and *Xenopus* nuclei that was not detected in studies where harsher fixation conditions were used (Cordes et al., 1993). Alternatively, the presence of CAN at the

cytoplasmic and nucleoplasmic fibrils could reflect CAN's ability to move through the pore with the transport complex.

It will be interesting to assess whether overexpressing other NPC proteins affects their sublocalization. The finding that some nucleoporins are present in different subregions of the NPC at distinct concentrations may be relevant to our understanding of the role of nucleoporins in nucleocytoplasmic transport.

Materials and Methods

Immunoelectron Microscopy. HeLa-derived cell line CE490S has been described previously (Fornerod et al., 1995). Cells were grown in the presence or absence of 1 μg/ml tetracycline, fixed with 2% paraformaldehyde in bicarbonate buffer (pH 7.4) for 90 min at room temperature, and stored in 1% paraformaldehyde in phosphate buffer (pH 7.3) at 4°C. Cells were scraped from the dish and pelleted in 10% gelatin. Ultrathin cryosectioning and immunolabeling were performed as described before (Fransen et al., 1991). Sections (~ 100 nm) were incubated with polyclonal αCNN antiserum (Fornerod et al., 1995), followed by protein A complexed to 10 nm gold particles, before being examined and photographed by using a JEOL JEM1010 electron microscope, operating at 80 kV.

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References

Corbett, A. H., Koepp, D. M., Schlenstedt, G., Lee, M. S., Hopper, A. K., and Silver, P. A. (1995). Rnalp, a Ran/TC4 GTPase activating protein, is required for nuclear import. J Cell Biol *130*, 1017-1713.

Cordes, V., Waizenegger, I., and Krohne, G. (1991). Nuclear pore complex glycoprotein p62 of Xenopus laevis and mouse: cDNA cloning and identification of its glycosylated region. Eur J Cell Biol *55*, 31-47.

Cordes, V. C., Reidenbach, S., Kohler, A., Stuurman, N., Van Driel, R., and Franke, W. W. (1993). Intranuclear filaments containing a nuclear pore complex protein. J Cell Biol *123*, 1333-1344.

Davis, L. I. (1995). The nuclear pore complex. Annu Rev Biochem *64*, 865-896.

Fabre, E., and Hurt, E. C. (1994). Nuclear transport. Curr Opin Cell Biol *6*, 335-342.

Fornerod, M., Boer, J., Van Baal, S., Jaeglé, M., Von Lindern, M., Murti, K. G., Davis, D., Bonten, J., Buijs, A., and Grosveld, G. (1995). Relocation of the carboxyterminal part of CAN from the nuclear envelope to the nucleus as a result of leukemia-specific chromosome rearrangements. Oncogene *10*, 1739-1748.

Fornerod, M., Boer, J., Van Baal, S., Morreau, H., and Grosveld, G. (1996). Interaction of cellular proteins with the leukemia specific fusion proteins DEK-CAN and SET-CAN and their normal counterpart, the nucleoporin CAN. Oncogene *13*, 1801-1808.

Fornerod, M., Van Deursen, J., Van Baal, S., Reynolds, A., Davis, D., Murti, K. G., and Grosveld, G. (1997). The human homologue of yeast CRM1 is in a dynamic subcomplex with CAN/Nup214 and a novel nuclear pore component Nup88. EMBO J *16*, 807-816.

Fransen, J. A. M., Hauri, H. P., Ginsel, L. A., and Naim, H. Y. (1991). Naturally occuring mutations in intestinal sucrase-isomaltase provide evidence for the existence of an intracellular sorting signal in the isomaltase subunit. J Cell Biol *115*, 45-57.

Görlich, D., Kraft, R., Kostka, S., Vogel, F., Hartmann, E., Laskey, R. A., Mattaj, I. W., and Izaurralde, E. (1996). Importin provides a link between nuclear protein import and U snRNA export. Cell *87*, 21-32.

Görlich, D., and Mattaj, I. (1996). Nucleocytoplasmic transport. Science *271*, 1513-1518.

Gossen, M., and Bujard, H. (1992). Tight control of gene expression in mammelian cells by tetracyclin-responsive promoters. Proc Natl Acad Sci USA *89*, 5547-5551.

Guan, T., Muller, S., Klier, G., Panté, N., Blevitt, J. M., Haner, M., Paschal, B., Aebi, U., and Gerace, L. (1995). Structural analysis of the p62 complex, an assembly of O-linked glycoproteins that localizes near the central gated channel of the nuclear pore complex. Mol Biol Cell *6*, 1591-1603.

Iovine, M. K., Watkins, J. L., and Wente, S. R. (1995). The GLFG repetitive region of the nucleoporin Nup116p interacts with Kap95p, an essential yeast nuclear import factor. J Cell Biol *131*, 1699-1713.

Izaurralde, E., and Mattaj, I. W. (1995). RNA export. Cell *81*, 153-159.

Kraemer, D., Wozniak, R. W., Blobel, G., and Radu, A. (1994). The human can protein, a putative oncogene product associated with myeloid leukemogenesis, is a nuclear pore complex protein that faces the cytoplasm. Proc Natl Acad Sci USA *91*, 1519-1523.

Melchior, F., and Gerace, L. (1995). Mechanisms of nuclear protein import. Curr Opin Cell Biol *7*, 310-318.

Melchior, F., Guan, T., Yokoyama, N., Nishimoto, T., and Gerace, L. (1995). GTP hydrolysis by Ran occurs at the nuclear pore complex in an early step of protein import. J Cell Biol *131*, 571-581.

Melchior, F., Paschal, B., Evans, J., and Gerace, L. (1993). Inhibition of nuclear protein import by nonhydrolyzable analogues of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor [published erratum appears in J Cell Biol 124:217, 1994]. J Cell Biol *123*, 1649-1659.

Moore, M. S., and Blobel, G. (1993). The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. Nature *365*, 661-663.

Moroianu, J., Hijikata, M., Blobel, G., and Radu, A. (1995). Mammalian karyopherin α1β and α2β heterodimers: α1 or α2 subunit binds nuclear localization signal and β subunit interacts with peptide repeat-containing nucleoporins. Proc Natl Acad Sci USA *92*, 6532-6536.

Nehrbass, U., and Blobel, G. (1996). Role of the nuclear transport factor p10 in nuclear import. Science *272*, 120-122.

Panté, N., and Aebi, U. (1993). The nuclear pore complex. J Cell Biol *122*, 977-984.

Panté, N., and Aebi, U. (1994). Toward the molecular details of the nuclear pore complex. J Struct Biol *113*, 179-189.

Panté, N., and Aebi, U. (1996). Toward the molecular dissection of protein import into nuclei. Curr Opin Cell Biol *8*, 397-406.

Panté, N., Bastos, R., McMorrow, I., Burke, B., and Aebi, U. (1994). Interactions and three-dimensional

localization of a group of nuclear pore complex proteins. J Cell Biol *126*, 603-617.

Radu, A., Blobel, G., and Moore, M. S. (1995a). Identification of a protein complex that is required for nuclear protein import and mediates docking of import substrate to distinct nucleoporins. Proc Natl Acad Sci USA *92*, 1769-1773.

Radu, A., Moore, M. S., and Blobel, G. (1995b). The peptide repeat domain of nucleoporin Nup98 functions as a docking site in transport across the nuclear pore complex. Cell *81*, 215-222.

Rexach, M., and Blobel, G. (1995). Protein import into nuclei: Association and dissociation reactions involving transport substrate, transport factors, and nucleoporins. Cell *83*, 683-692.

Rout, M. P., and Wente, S. R. (1994). Pores for thought: Nuclear pore complex proteins. Trends Biochem Sci *4*, 357-363.

Schlenstedt, G. (1996). Minireview: Protein import into the nucleus. FEBS Lett *389*, 75-79.

Schlenstedt, G., Saavedra, C., Loeb, J. D., Cole, C. N., and Silver, P. A. (1995). The GTP-bound form of the yeast Ran/TC4 homologue blocks nuclear protein import and appearance of poly(A)+ RNA in the cytoplasm. Proc Natl Acad Sci USA *92*, 225-229.

Sukegawa, J., and Blobel, G. (1993). A nuclear pore complex protein that contains zinc finger motifs, binds DNA, and faces the nucleoplasm. Cell *72*, 29-38.

Van Deursen, J., Boer, J., Kasper, L., and Grosveld, G. (1996). G_2 arrest and impaired nucleocytoplasmic transport in mouse embryos lacking the proto-oncogene *CAN/Nup214*. EMBO J *15*, 5574-5583.

Wente, S. R., and Blobel, G. (1994). NUP145 encodes a novel yeast glycine-leucine-phenylalanine-glycine (GLFG) nucleoporin required for nuclear envelope structure. J Cell Biol *125*, 955-969.

Wente, S. R., and Blobel, G. (1993). A temperature-sensative NUP116 null mutant forms a nuclear envelope seal over the yeast nuclear pore complex thereby blocking nucleocytoplasmic traffic. J Cell Biol *123*, 275-284.

Wilken, N., Senécal, J.-L., Scheer, U., and Dabauvalle, M.-C. (1995). Localization of the Ran-GTP binding protein RanBP2 at the cytoplasmic side of the nuclear pore complex. Eur J Cell Biol *68*, 211-219.

Wu, J., Matunis, M. J., Kraemer, D., Blobel, G., and Coutavas, E. (1995). Nup358, a cytoplasmically exposed nucleoporin with peptide repeats, Ran-GTP binding sites, zinc fingers, a cyclophilin A homologous domain, and a leucine-rich region. J Biol Chem *270*, 14209-14213.

Yokoyama, N., Hayashi, N., Seki, T., Panté, N., Ohba, T., Nishii, K., Kuma, K., Hayashida, T., Miyata, T., Aebi, U., Fukui, M., and Nishimoto, T. (1995). A giant nucleopore protein that binds Ran/TC4. Nature *376*, 184-188.

Interaction of cellular proteins with the leukemia specific fusion proteins DEK-CAN and SET-CAN and their normal counterpart, the nucleoporin CAN

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Interaction of cellular proteins with the leukemia specific fusion proteins DEK-CAN and SET-CAN and their normal counterpart, the nucleoporin CAN

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The recurrent chromosomal translocation (6;9) is associated with acute myeloid leukemia and results in expression of the DEK-CAN fusion protein. This oncoprotein consists of almost the entire DEK protein fused to the C-terminal two-thirds of the CAN protein. In much the same way, CAN is fused to SET in a patient with acute undifferentiated leukemia, producing a SET-CAN fusion protein. Interestingly, CAN is associated with the nuclear pore complex (NPC) and we recently established its crucial role in nucleocytoplasmic transport processes and cell cycle progression. As a first step in the biochemical analysis of the oncogenic mechanism associated with translocation (6;9), we set out to identify proteins that interact with CAN and its fusion proteins. We found that two proteins specifically co-immunoprecipitate with CAN. One had a molecular mass of 88 kDa protein (CC88) and was determined to associate with the central region of CAN that contains several protein interaction motifs. A second protein of 112 kDa (CC112) was found to interact with the C-terminal nucleoporin-specific repeat of CAN, a region that is supposed to function in nucleocytoplasmic transport. CC112 also interacts with the DEK-CAN and SET-CAN fusion proteins. This finding suggests that CC112 may contribute an essential function to the leukemogenic effect of DEK-CAN and SET-CAN.

Keywords: DEK-CAN; SET-CAN; *can* proto-oncogene; nucleoporins; acute leukemia

Introduction

The recurrent translocation (6;9)(p23;q34) is associated with human myeloid leukemia of diverse phenotype (Rowley and Potter, 1976; Soekarman *et al.*, 1992). It is often the sole karyotypic abnormality found in the leukemic cells, which suggests a causative role for this translocation in the disease. The translocation creates a *dek-can* fusion gene on the 6p$^-$ chromosome (von Lindern *et al.*, 1990, 1992a), that is predicted to encode a structurally identical 165 kDa DEK-CAN chimeric protein in all patients analysed. This protein contains all but the 26 C-terminal amino acids of DEK linked to the C-terminal two-thirds of CAN. In addition, we found that a cryptic translocation or inversion of chromosome 9 in a patient with acute undifferentiated leukemia, created a chimeric protein comprising the same sequences of CAN but linked to N-terminal sequences of SET (von Lindern *et al.*, 1992b).

The CAN protein was found to belong to a family of nuclear pore complex associated proteins called nucleoporins (Fornerod *et al.*, 1995; Kraemer *et al.*, 1994). This group of proteins is thought to play an important role in transport of RNA and protein across the nuclear membrane (for review see Görlich and Mattaj, 1996; Rout and Wente, 1994). In HeLa cells, CAN is found generally at the fibrillar, cytoplasmic extrusions of the nuclear pore complex (NPC) (Kraemer *et al.*, 1994; Panté *et al.*, 1994), but is also detected at the nuclear face upon over-expression (Fornerod *et al.*, 1995, JB and GG, unpublished results). CAN is also referred to as NUP214 (Kraemer *et al.*, 1994) or p250 (Panté *et al.*, 1994).

Recently, we created a CAN knockout mutation in the mouse (van Deursen *et al.*, 1996) and found that CAN$^{-/-}$ embryos survive until day 4 of gestation by using maternal, oocyte-derived protein. At this point in development, the dwindling amounts of CAN become critical, resulting first in a G2 arrest of embryonic cells, followed by a detectable decrease in nuclear localization signal (NLS)-mediated protein import that coincides with a block in mRNA export. Although these data firmly establish a role for CAN in nucleocytoplasmic transport across the nuclear pore complex, they shed little light on CAN's contribution to leukemogenic transformation.

Presently the cellular function of the DEK protein is not known. It is a nuclear protein that may be associated with chromatin (Fornerod *et al.*, 1995). The SET protein is also a nuclear protein and was recently identified as a replication factor TAF-1 (Nagata *et al.*, 1995). It appears to be part of a growing superfamily of proteins, including the yeast nucleosome assembly factor NAP1 (Nagata *et al.*, 1995; von Lindern *et al.*, 1992b), that specifically interact with B-type cyclins (Kellogg *et al.*, 1995). How these features should be interpreted in the context of leukemogenesis remains to be established. DEK has no sequence homology with SET, apart from highly acidic regions that are present in both proteins.

The primary amino acid sequence of CAN contains several distinct motifs including 11 FXF-sequence repeats scattered throughout the molecule, and a 35x repeated FG sequence that is confined to the C-terminus (von Lindern *et al.*, 1992a). Similar repeat sequences appear in several vertebrate and yeast nucleoporins (for review see Rout and Wente, 1994), and it has been suggested that these repeats are involved in protein-protein interactions that mediate substrate transport through the NPC (Chi *et al.*, 1995; Görlich *et al.*, 1995; Moroianu *et al.*, 1995; Radu *et al.*,

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1995a). In fact, *in vitro* studies have shown that the 97 kDa import factor p97/importin-β subunit of the nuclear protein import complex binds to the repeat-containing nucleoporins NUP98, NUP153, NUP358/RanBP2, and CAN (Moroianu *et al.*, 1995; Radu *et al.*, 1995b). CAN also contains a coiled-coil region and a leucine zipper, situated at the N-terminal side of the translocation breakpoint, and a second coiled-coil region that flanks the breakpoint at the C-terminal side. These two domains are part of the central region of CAN that is responsible for nuclear envelope association (Fornerod *et al.*, 1995), and therefore could mediate NPC interaction. Surprisingly, mutants of CAN lacking these domains are directed to the nucleus. The sequences mediating this relocation are contained within the C-terminal 227 amino acids of the molecule (Fornerod *et al.*, 1995).

The DEK-CAN and SET-CAN fusion proteins retain one of the central coiled-coil domains and the FG repeat sequences. In contrast to CAN, these fusion proteins are permanently located in the nucleus and therefore we hypothesized that permanent relocation of CAN sequences to the nucleus could contribute an essential component to their leukemogenic activity. Hence, proteins that would physically interact with this portion of CAN may convey or contribute to the transforming potential of the fusion proteins.

Recently, a second nucleoporin was found to be targeted by a recurrent translocation in acute myeloid leukemia. This translocation (7;11) creates a fusion between the FXFG-repeat containing part of nucleoporin NUP98 and the homeobox-containing C-terminal part of HoxA9 (Borrow *et al.*, 1996; Nakamura *et al.*, 1996). This discovery suggests that the mode of action of the CAN and NUP98 derived fusion proteins could be related, and emphasizes the need for detailed molecular analyses of leukemia-associated nucleoporins.

Here, as a further step to addressing the contribution of CAN to leukemogenesis, we define proteins that co-immunoprecipitate with CAN. We also identify the domains in CAN that interact with these proteins. Only one of the proteins appears to bind to DEK-CAN and SET-CAN and we believe this protein may be involved in the transformation process of myeloid precursor cells.

Results

Co-immunoprecipitation of CAN interacting proteins

We performed co-immunoprecipitation studies to identify CAN, DEK-CAN or SET-CAN interacting proteins, that could be important for their function. Our polyclonal CAN antisera (Fornerod *et al.*, 1995) only effectively immunoprecipitated the protein in the presence of SDS (data not shown), thereby dissociating pre-existing protein complexes. We overcame this problem by using previously generated expression plasmids encoding influenza virus hemagglutinin (HA1)-tagged versions of CAN and DEK-CAN (HA1-CAN and HA1-DEK-CAN) (Fornerod *et al.*, 1995). HA1-CAN and HA1-DEK-CAN were transiently expressed in the HeLa derived cell-line HtTA-1 (Gossen and Bujard, 1992) and the proteins were subsequently immunoprecipitated with anti-HA1 monoclonal 12CA5 (Wilson *et al.*, 1984). As shown in Figure 1, two major proteins are co-precipitated with HA1-CAN from ^{3}H-leucine labeled cells; one of 112 and one of 88 kDa. A weaker band representing a protein of 66 kDa can also be observed. We named these proteins CC112, CC88 and CC66 (for CAN Co-precipitating protein) and found that only CC112 co-precipitated with HA1-DEK-CAN (Figure 1) and HA1-SET-CAN (not shown). These data suggest that CC112 interacts with the C-terminal part of CAN, whereas CC88 and CC66 require sequences that are either disrupted by the fusion or are no longer present in DEK-CAN and SET-CAN. To confirm this interpretation, we separately expressed HA1-tagged versions of the N-terminal half of CAN (amino acids 1–1058), and the C-terminal half (amino acids 1059–2090). Indeed, the N-terminal half of CAN co-precipitated CC88 and CC66, while the C-terminal part of CAN co-precipitated only CC112 (Figure 1). No smaller co-precipitating proteins were detected in HtTA-1 cells (not shown).

To ensure that expression of these co-precipitating proteins was not limited to HtTA-1 cells, we performed a similar co-immunoprecipitation experiment using human U937 monoblastic cells, engineered to express HA1-CAN in a tetracycline dependent manner (cell line U937T-C7). We cultured these cells in the presence of different concentrations of tetracycline to establish distinct expression levels of HA1-CAN. As shown in Figure 2, CC112 and CC88 were also co-precipitated with HA1-CAN expressed in U937T-C7, indicating that, similar to CAN (von Lindern *et al.*, 1992a), these proteins are expressed in multiple human cell types. The signal of ^{35}S-labeled CC88 was relatively weak,

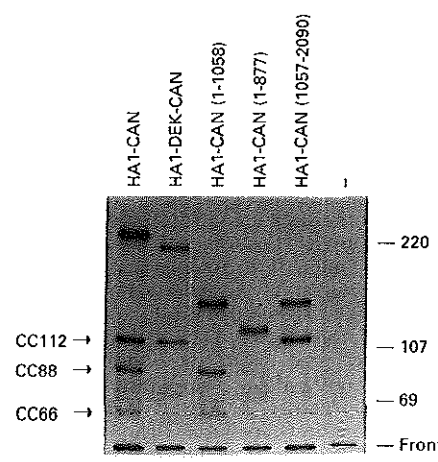

Figure 1 CAN co-immunoprecipitating proteins in HeLa derived HtTA-1 cells. Cells were transfected with expression plasmids encoding HA1 tagged versions of the CAN and DEK-CAN proteins or CAN deletion mutants as indicated above the lanes. Untransfected cells are represented in the right lane (–). Following metabolic labeling with [^{3}H]leucine, proteins were immunoprecipitated using monoclonal antibody 12CA5 and analysed on a 6% polyacrylamide gel. Co-precipitating proteins are indicated by arrows on the left. Molecular masses of standard proteins are indicated on the right (kDa)

which we found is typical for ^{35}S-methionine labeling of this protein. Remarkably, the amount of co-precipitating CC88 remained more or less constant irrespective of the cellular HA1-CAN concentration. In contrast, the amount of co-precipitated CC112 increased proportionally to the amounts of HA1-CAN expression. These data suggest that there is a fixed amount of CC88 in the cell available for co-precipitation, whereas there is a surplus of CC112 of which normally only a portion is bound to CAN. A co-precipitating protein corresponding in size to CC66 in HeLa cells was also seen in U937T-C7 cells. Like CC112, the amount of CC66 increased proportionally with the amount of HA1-CAN.

Since CC112 and CC88 appeared to be cellular proteins that specifically interact with nucleoporin CAN, they could provide important new insights into the function of CAN, DEK-CAN and SET-CAN. We studied their interaction with CAN in detail and generated a collection of plasmids encoding HA1-CAN deletion mutants (see Materials and methods). After transient transfection into HtTA-1 cells, ^{3}H-labeled proteins were immunoprecipitated with 12CA5, and analysed on 6% SDS–PAGE gels. Figure 3 summarizes the results of this analysis.

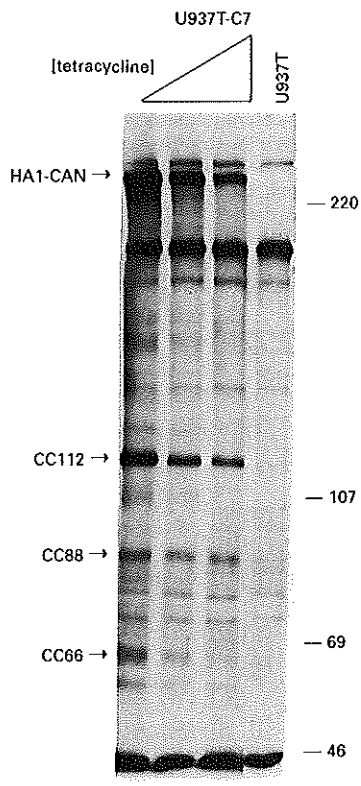

Figure 2 CAN co-immunoprecipitating proteins in human myeloid cell line U937T. HA1-CAN expression is tetracycline repressible in the stably transfected cell line U937T-C7. Cells were grown in 4,6 or 10 ng/ml tetracycline (from left to right). Parental cell line U937T that does not express HA1-CAN is represented in the right lane. Following metabolic labeling with [^{35}S]methionine, proteins were immunoprecipitated using monoclonal antibody 12CA5 and analysed on a 6% polyacrylamide gel. HA1-CAN and the co-precipitating proteins are indicated by arrows on the left. Molecular masses of standard proteins are indicated on the right (kDa)

CC112 co-precipitation

The deletion analysis shows that the smallest region in the C-terminal half of CAN that could mediate co-immunoprecipitation of CC112 consisted of the C-terminal 227 amino acids (Figure 3; CAN 1864–2090). This represents the C-terminal portion of the FG repeat of CAN. Interestingly, the N-terminal half of this repeat, represented by CAN 1558–1839, was not able to co-precipitate CC112. This indicated that the FG repeat region of CAN is heterogenous in nature, an observation not made before for nucleoporin repeat regions. To map the CC112 binding region within the last 227 amino acids, we tested additional N-terminal (CAN 1957–2090), C-terminal (CAN 1864–2052) and internal (CAN 1864–1912, 1984–2090) deletion mutants (Figure 3). None of these proteins was able to co-precipitate CC112, suggesting that strong CAN/CC112 interaction requires the entire C-terminal FG repeat region.

Since association with CC112 could represent an important function of CAN, DEK-CAN and SET-CAN, we investigated naturally occurring alternative forms of its binding region. Screening for alternatively spliced *can* mRNAs by RT–PCR, led us to identify a cDNA that represents a minor transcript in KG-1 and HeLa cells that lacks the exon representing nucleotides 5687–5843 of the *can* mRNA, encoding amino acids 1865–1916 (data not shown). At codon 1917, translation of the alternatively spliced mRNA changes to a different reading frame that remains open until position 6519, 154 nucleotides downstream of the normal stop codon. The 224 amino acid sequence encoded by the alternative reading frame shows no significant homology to known proteins, and does not contain any nucleoporin repeats. When this alternatively spliced form was introduced into the normal *can* cDNA, the expressed protein (CAN FS1865, see Figure 3) was unable to co-precipitate CC112, whereas CC88 precipitation was unaffected.

CC88 co-precipitation

Unlike CC112, co-precipitation of CC88 required the N-terminal half of CAN (Figure 1). CAN mutant 586–1058 was still able to co-precipitate CC88, whereas the mutant lacking 709–1094 (CAN1-708, 1095–2090) did not, further delineating CC88 interacting sequences to CAN's central region. The same region was previously identified to be important for association of CAN with the nuclear envelope and, by implication, the nuclear pore (Fornerod *et al.*, 1995). We therefore wished to map this region of interaction in more detail. The central domain of CAN contains three potential protein-protein interaction domains (Fornerod *et al.*, 1995; von Lindern *et al.*, 1992a). A predicted N-terminal coiled-coil region and an adjacent leucine zipper are contained within amino acids 702–774, flanked by a second, more C-terminally located coiled-coil region formed by amino acids 860–887.

CAN deletion mutants that lack the N-terminal coiled-coil and leucine zipper region, such as CAN 1–708, 816–2090 and CAN 804–1085 failed to co-precipitate CC88, establishing the importance of this region for CC88 interaction. The same was found for mutants that lack (CAN 1–832) or truncate (CAN 1–877, Figure 1) the second coiled-coil region. These data indicate that multiple putative interaction domains contribute to CC88 binding and are needed for co-precipitation.

To further investigate whether the N-terminal coiled-coil, the leucine zipper and/or the C-terminal coiled-coil are involved in CC88 interaction, we introduced point mutations in these motifs using the CAN mutant 586–1085 background. In all cases, aliphatic amino acids in heptad repeat motifs of predicted α-helices were exchanged for helix-breaking prolines (Figure 4a). Since proteins had been metabolically labeled with ^{35}S-methionine and the number of methionines remained constant in these CAN mutants, quantitation of the ratio of radioactivity incorporated in immunoprecipitated CAN and CC88 directly reflected their binding affinity. As shown in Figure 4b, mutation of $I_{754}\rightarrow P$ strongly diminished co-precipitation of CC88, suggesting that the leucine zipper of CAN is important for CC88 binding. By contrast, no change in the amount of co-immunoprecipitating CC88 could be detected when prolines were introduced into the N-terminal ($I_{710}\rightarrow P$) or C-terminal ($V_{875}\rightarrow P$) coiled-coil regions. The double mutation $I_{710}\rightarrow P + V_{875}\rightarrow P$ did cause a marked decrease in CC88 co-precipitation. This finding is in agreement with our deletion analysis; CC88 binding involves both the N- and C-terminal α-helical protein interaction regions of CAN's central domain.

Subcellular localization of CAN mutants

The C-terminal FG-repeat region of CAN required for CC112 co-immunoprecipitation (amino acids 1864–2090) coincides with the region previously identified to confer nuclear localization of CAN mutants that do not associate with the nuclear pore (Fornerod *et al.*, 1995). Similarly, amino acids 586–1058 of CAN required for CC88 co-immunoprecipitation coincide with a region shown to be necessary and sufficient for nuclear pore association. To further investigate these apparent relations, we determined the subcellular localization of the CAN mutants described above by indirect immunofluorescence.

Expression of the N-terminal half of the C-terminal FG repeat (amino acids 1558–1839; Figure 3), that did not co-immunoprecipitate CC112, showed that this protein is located exclusively in the cytoplasm. This result identified the C-terminal 227 amino acids of CAN as both necessary and sufficient for nuclear localization, and further sustained the correlation between nuclear localization and CC112 co-precipitation. However, deletion mutants within the C-terminal, CC112-binding half of the FG repeat (Figure 3) were either completely or partially located to the nucleus, but did not co-precipitate CC112.

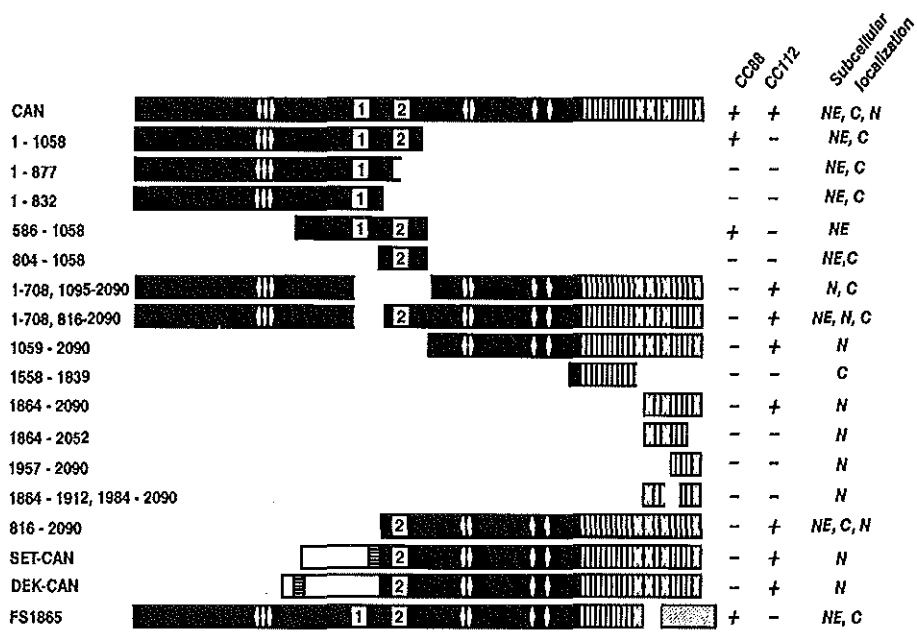

Figure 3 Mapping of domains in CAN mediating co-precipitation of CC88 and CC112 and subcellular localization. Bars represent CAN and CAN mutant proteins; numbers on the left represent amino acid boundaries; FS, frame-shift. The ability of mutants to co-immunoprecipitate CC88 or CC112 is indicated by pluses and minuses on the right hand side. Subcellular localization: NE, nuclear envelope; C, cytoplasm; N, nucleus. Multiple localizations appear in order of prominence. Predicted structural motifs are represented as follows: FG repeats as vertical bars, FXF repeats as diamonds, α-helical protein interaction domains as white boxes: (1) coiled-coil#1 and adjacent leucine zipper; (2) coiled-coil#2. The cross-hatched box represents the alternative reading frame in CAN FS1865. Horizontal stripes indicate acidic regions in the DEK and SET proteins

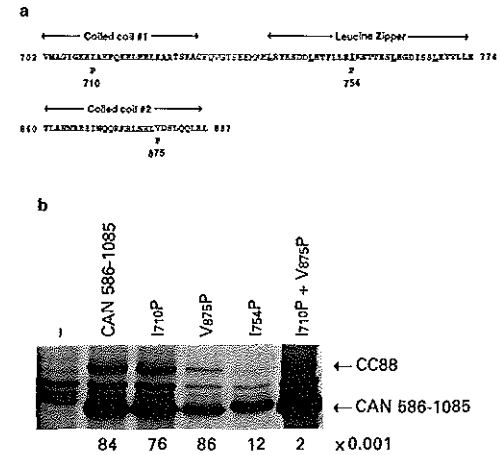

Figure 4 Effect of point mutations in predicted α-helical protein interaction domains of CAN on CC88 co-immunoprecipitation. (a) Amino acid sequence of the two predicted α-helical protein interaction domains flanking the t(6;9) breakpoint. Arrows are placed above coiled-coil#1, the leucine zipper and coiled-coil#2. (Iso)leucines that form the leucine zipper are underlined. Point mutations introduced are indicated underneath the sequence. (b) HtTA-1 cells were transfected with expression plasmids encoding HA1-CAN 586–1085 derivatives, carrying point mutations as indicated above the lanes. Untransfected cells are represented in the left lane (−). Following metabolic labeling with [^{35}S]methionine, proteins were immunoprecipitated using monoclonal antibody 12CA5 and analysed on a 6% polyacrylamide gel. HA1-CAN 586–1085 and CC88 are indicated by arrows on the right. The ratio of radioactivity present in CC88 (×1000) over that of CAN 586–1085 as determined by phosphorimager analysis is shown below each lane

Immunofluorescence analysis of the central domain of CAN showed that mutants that have either the N- or the C-terminal predicted protein interaction domains intact, such as CAN 804–1085 and CAN 1–832, can be detected at the nuclear envelope, even if they do not co-precipitate CC88. Nuclear envelope association of mutants missing the C-terminal coiled-coil (CAN 1–832) was decreased. Similarly, coiled-coil point mutant V_{875}→P displayed a marked decrease in nuclear envelope localization, and double mutant I_{710}→P+V_{875}→P hardly showed any association at all (data not shown). In contrast, the single coiled-coil point mutation I_{710}→P did not noticeably decrease the protein's ability to associate with the nuclear envelope, nor did leucine zipper point mutation I_{754}→P. These data show that localization of CAN at the nuclear envelope does not correlate with CC88 co-precipitation, and they identify the C-terminal coiled-coil as the most important motif for nuclear pore association.

DEK-CAN and SET-CAN are nuclear proteins, in effect relocating the C-terminal part of CAN from the nuclear pore to the nucleoplasm (Fornerod *et al.*, 1995). Thus, we suspected that other sequences in the fusion proteins override the nuclear envelope interaction mediated by amino acids 813–1058. To test whether this is dependent on nuclear localization signals in DEK and SET, we expressed the CAN part (CAN 816–2090) of the two fusion proteins alone. This mutant predominantly located to the nuclear envelope (Figure 3), demonstrating that DEK-CAN and SET-CAN depend on their DEK and SET moieties for relocation to the nucleus.

Discussion

As a first step to biochemically analyse the contribution of CAN sequences to the transforming capacity of the DEK-CAN and SET-CAN fusion proteins, we identified a 112 kDa protein (CC112) that associates with the C-terminal nucleoporin-repeat of CAN, DEK-CAN and SET-CAN. In addition, we found that the central region of CAN binds to an 88 kDa protein (CC88), that does not co-immunoprecipitate with the fusion proteins.

Using CAN mutants, we were able to locate regions in the CAN protein responsible for interaction with CC112 and CC88. Since these regions operate independently and CC112 and CC88 are by far the most prominent co-precipitating proteins, binding is likely to be direct. However, we cannot exclude that association of either of the two proteins is mediated by other proteins that escaped our attention, for instance due to their small size. We noticed less consistent co-immunoprecipitation of a 66 kDa protein (CC66) in both HeLa and U937 derived cells. Because of this inconsistency, most likely caused by weaker binding to CAN and/or CC88, we decided to focus our analysis on the 88 and 112 kDa proteins.

CC112/CAN interaction

The region of CAN necessary and sufficient for CC112 co-precipitation consists of the C-terminal 227 amino acids. This region represents the distal half of CAN's 420 amino acid nucleoporin specific FG-repeat. We cannot identify significant differences in either FG density or inter-FG amino acid composition between these two halves of the FG repeat. It is therefore unclear what structural features of the distal part constitute the CC112 binding specificity. Identification of the FG repeat as a protein interaction domain *per se* suggests that its N-terminal part could specifically interact with other cellular proteins that may bind weaker than CC112, or that are less abundant in HeLa and U937 cells. In this respect, it is noteworthy that we isolated a cDNA for an alternatively spliced *can* mRNA encoding a C-terminally truncated FG-repeat. A CAN protein carrying this shortened repeat region could be functionally distinct since it displays a different specificity for associating factors, illustrated by the fact that it does not co-precipitate CC112.

The N-terminal half of the FG repeat alone localizes to the cytoplasm, whereas the C-terminal half localizes to the nucleus. This C-terminal domain of CAN bears no homology to known nuclear localization signals, such as a standard (Kalderon *et al.*, 1984) or bipartite NLS (Robbins *et al.*, 1988) or the M9 sequence of hnRNPA1 (Siomi and Dreyfuss, 1995). Thus, unless the C-terminal repeat of CAN carries an as yet unrecognized NLS-type sequence, transport to the nucleus must be mediated by an associating nucleophilic protein. An obvious candidate for this would be CC112, except for the apparently conflicting observation that additional N-terminal, C-terminal and

internal deletion mutants of the C-terminal FG repeat still localize to the nucleus, but do not co-precipitate CC112. However, in these mutants, CC112 binding *in vivo* may be strong enough to mediate nuclear transport but too weak to withstand our co-immunoprecipitation conditions. Thus, resolving the question whether CC112 mediates the nuclear localization of C-terminal CAN awaits the molecular cloning of CC112. This would allow us to use more sensitive methods to probe for interaction between these proteins, such as a two-hybrid analysis in yeast.

Since CC112 interacts with CAN, which is part of the nuclear pore complex, CC112 is likely to be present at the nuclear pore as well. Our data strongly indicate that there is a molar excess of CC112 as compared to CAN in the cell. This additional CC112 may be bound to other nuclear pore components, in particular to those containing FG repeat sequences, or it may be located at additional sites in the cell. Since DEK-CAN and SET-CAN are nuclear proteins, we expect that CC112 is also located in the nucleus.

CC88/CAN interaction

A second co-immunoprecipitating protein, CC88, interacts with the central region of CAN containing three potential protein interaction domains: two coiled-coil regions and a leucine zipper (Fornerod *et al.*, 1995; von Lindern *et al.*, 1992a). Coiled-coil domains are common motifs in nuclear pore components, such as NUP82 (Grandi *et al.*, 1995), Trp (Byrd *et al.*, 1994), NSP49 (Hurt, 1989), p62 (Starr *et al.*, 1990) and Nsp1 (Wimmer *et al.*, 1992). Their coiled-coil domains appear to be involved in association of these proteins in nuclear pore subcomplexes (Grandi *et al.*, 1995) and could also be required for anchoring to structural components of the nuclear pore (Byrd *et al.*, 1994; Hurt, 1989). Our data show that the coiled-coil regions of CAN are prime candidates to mediate binding of this nucleoporin to the NPC, since point mutations in these motifs negatively influence CAN's nuclear envelope localization.

CC88 co-precipitation is drastically decreased by a single point mutation in the leucine zipper as well as by combined point mutations in both coiled-coils. These data indicate that strong interaction required for co-immunoprecipitation is mediated by all three α-helical interaction domains. This notion is also in agreement with our deletion analysis that shows that mutants that lack any of these domains are unable to co-precipitate CC88. We cannot exclude, however, the possibility that the point mutations affect normal folding of a larger region that could include other CC88 binding domains.

All CAN mutants that interact with CC88 are predominantly located at the nuclear envelope, and by implication at the nuclear pore. It is therefore likely that CC88 is part of the nuclear pore complex. Because over-expression of CAN does not increase the amount of co-precipitating CC88, the NPC may be its only location. This raises the question as to whether the apparent lack of CC88 interaction with DEK-CAN and SET-CAN could be the result of differential cellular compartmentalization. We find this explanation unlikely since CAN mutants that are located at the nuclear envelope (such as CAN 804–1058 or CAN 816–2090), and contain the same part of CAN's central region as DEK-CAN or SET-CAN, do not co-precipitate CC88 either. Thus, at present we have no indications that CC88 could contribute to the DEK-CAN or SET-CAN mediated leukemic process.

Concluding remarks

We have shown that CC112 interacts with DEK-CAN and SET-CAN. Insights into the functional significance of this 112 kDa protein for the leukemic process, is obviously hindered by our lack of understanding of the function of DEK-CAN and SET-CAN themselves. However, we would like to present two hypothetical scenarios.

(i) The gene fusion between NUP98 and HoxA9 has recently been described in leukemic patients carrying translocation (7;11) (Borrow *et al.*, 1996; Nakamura *et al.*, 1996). Interestingly, the part of NUP98 that is predicted to be present in the NUP98/HoxA9 fusion protein consists of its nucleoporin-specific FXFG repeat, whereas sequences contributed by the transcription factor HoxA9 include its DNA binding domain. If NUP98-HoxA9 and DEK-CAN/SET-CAN work in a similar fashion, DEK and SET may contribute a DNA binding property to our fusion proteins. The observations that DEK co-localizes with metaphase chromosomes in mitotic cells (Fornerod *et al.*, 1995) and binds to double-stranded DNA *in vitro* (Titia de Lange, personal communication) could support this idea. SET has been shown to stimulate adenovirus replication *in vitro* (Nagata *et al.*, 1995) and thus could also be involved in DNA binding. Therefore, in analogy to many other oncogenic nuclear fusion proteins (for review see Rabbitts, 1994), these nucleoporin fusions may act as mutant transcription factors, where DEK, SET or HoxA9 would mediate DNA binding to regulatory sequences of target genes, whereas CAN or NUP98 provide sequences essential for transcriptional disregulation. Thus proteins that associate with the nucleoporin portions of these fusion proteins, such as CC112, may provide an essential contribution.

(ii) Another, but not mutually exclusive possibility is, that nucleoporin related fusion proteins deregulate cellular growth through changes in nucleocytoplasmic transport. A protein was recently described, that is involved in Rev-mediated export of HIV RNA from the nucleus (Bogard *et al.*, 1995; Friz *et al.*, 1995; Stutz *et al.*, 1995). This protein, called Rab or RIP, shows significant homology to the CC112-interaction domain of CAN. We find this homology fascinating, particularly in view of our observation that CAN is essential for nucleocytoplasmic transport of protein and mRNA (van Deursen *et al.*, 1996). In addition to the viral Rev protein, one cellular protein has previously been shown to interact with nucleoporin-specific repeats: import factor p97/importinβ. This protein is part of a cytoplasmic complex that mediates transport of NLS containing proteins from the cytoplasm to the nuclear pore (see for instance Görlich and Mattaj, 1996). In analogy to Rev and p97/importinβ, CC112 may be involved in transport processes between the nuclear pore and the nucleoplasm and/or cytoplasm, that may be disturbed by fusion proteins such as DEK-CAN and SET-CAN.

We hypothesize, that further analysis of the CC112 protein may provide crucial information to improve our understanding of how DEK-CAN and SET-CAN

deregulate growth of early hematopoietic progenitor cells. Further analysis of CC112 and CC88 may also shed more light on CAN's function in nucleocytoplasmic transport.

Materials and methods

Expression constructs

All expression plasmids used in this study carry sequences encoding two copies of the influenza virus hemagglutinin (HA1) tag at the 5' end of their open reading frames (Fornerod *et al.*, 1995). All constructs with the exception of pSCTOP-HA1-Set-Can, make use of the Tet-VP16 responsive promoter (Gossen and Bujard, 1992). SET-CAN expression was directed by plasmid pSCTOP-HA1-Set-Can. This plasmid was constructed by replacing the *set* insert of pSCTOP-HA1-Set (Fornerod *et al.*, 1995) by a *Sac*I *set-can* fragment from anti-sense pCDXX-Set-Can (Fornerod *et al.*, 1995). DEK-CAN expression was directed by plasmid pHA1-Dek-Can (Fornerod *et al.*, 1995). pHA1-Can is similar to pCDXX-HA1-Can (Fornerod *et al.*, 1995), with the exception that the *Eco*RI cDNA insert is present in the pUHD10S vector (Fornerod *et al.*, 1995). Expression of deletion mutants CAN 1–1058 and CAN 586–1058 was directed by plasmids pHA1-CANΔ 1059–2090 and pHA1-CANΔ 1–585, and Δ 1059–2090 respectively (Fornerod *et al.*, 1995). pHA1-CAN 1–832 was created by filling in the *Xba*I site of pHA1-CANΔ 1059–2090 at position 2587 of the *can* cDNA (von Lindern *et al.*, 1992a), creating a stop after codon 832. pHA1-CAN 1864–2090 was derived from pHA1-CANΔ 1–1139, Δ 1341–1863 (Fornerod *et al.*, 1995) by deleting sequences encoding amino acids 1140–1340 using the *Nhe*I site flanking the HA1 encoding region and the *Msc*I site at position 5680 of the *can* cDNA. pHA1-CAN 1864–2052 is a truncation derivative of this plasmid using *Xcm*I sites at position 6244 of the *can* cDNA and in pUHD10S. pHA1-CAN 1957–2090 was also derived from pHA1-CAN 1864–2090 by deleting sequences between the *Nco*I site flanking the HA1 tag coding region and the *Sty*I site at position 5758 of the *can* cDNA. pHA1-CAN 1864–1912, 1984–2090 was created by an in frame deletion using the *Bam*HI sites at positions 5825 and 6044 of the *can* cDNA. pHA1-CAN 1556–1839 was derived from pHA1-Can by ligation of the filled-in *Nde*I site flanking the HA1 tag coding region onto the *Spe*I site at position 4766 of the *can* cDNA and subsequent filling in of the HindIII site at position 5607. This creates a stop 5 codons downstream of codon 1839. pHA1-CAN 816–2090 was derived from pHA1-Can by deleting sequences upstream of the *Kas*I site at position 2538 of the *can* cDNA. Deletions in expression plasmids pHA1-CAN 1–877, pHA1-CAN 1–1085, pHA1-CAN 804–2090 and pHA1-CAN 1059–2090 were created using partial *E. coli* exonucleaseIII digestion using the Erase-a-base system (Promega, Madison, WI Henikoff, 1984) and was performed according to manufacturers' directions. For this purpose, two modifications of pHA1-Can were constructed. For N-terminal deletions, a *Not*I–*Sph*I linker was placed between sequences encoding the HA1 tag and the *Nde*I site at amino acid 1 of the *can* open reading frame. Removal of nucleotides originated from the *Nde*I site, while the 3' overhanging *Sph*I end protected upstream sequences from exo III digestion. Reading frames could be corrected by introducing adapters into the unique *Not*I site. For C-terminal deletions, a *Sal*I–*Sph*I–*Not*I linker was introduced 3' of the *can* cDNA, and removal of nucleotides started at the *Sal*I site, whereas the *Sph*I 3' overhanging end prevented downstream deletions. N- and C-terminal deletions could be linked using *Not*I sites, giving rise to internal CAN deletion mutants such as pHA1-CAN 1–708, 1095–2090. pHA1-CAN 586–1085 was created by an N-terminal truncation of pHA1-CAN 1–1085 using a *Hinc*II site as described (Fornerod *et al.*, 1995). pHA1-CAN 804–1058 was constructed by combining pHA1-CAN 804–2090 and pHA1-CANΔ 1059–2090. In a similar way, pHA1-CAN 1–708, 816–2090 is the combination of pHA1-CAN 1–708 and pHA1-CAN 816–2090. Point mutations in pHA1-CAN 586–1085 were generated in bacteriophage M13, using a commercial kit (Muta-Gene, BioRad, Hercules, CA) based on the Kunkel method (Kunkel, 1985). The following proline codons were introduced: amino acid 710(I→P): CCG, 754(I→P): CCT, 825(V→P): CCC.

Cell culture and transfection

HtTA-1 culture and transfection was performed as previously described (Fornerod *et al.*, 1995). Cell line U937T is a derivative of the human monoblast cell line U937 (Sundström and Nilsson, 1976) that expresses the tetracycline responsive chimeric transactivator Tet-VP16 (Gossen and Bujard, 1992). U937T-C7 is stably transfected with pHA1-Can. A complete description and analysis of this cell line will be published elsewhere (JB and GG, manuscript in preparation).

Immunoprecipitation and immunofluorescence

2×10^5 cells grown in 6 cm dishes were metabolically labeled for 6–16 h using 100 μCi [^{3}H]leucine (Amersham Corp., Arlington Heights, IL) or [^{35}S]methionine/cysteine *in vivo* labeling mix (DuPont NEN, Wilmington, DE) in 1.4 ml of leucine or methionine free DMEM, supplemented with 8% dialyzed fetal calf serum. Metabolic labeling was carried out in a closed container, in order to avoid radioactive contamination of the environment (Fornerod, 1996). Forty to fifty hours after transfection, cells were cooled on ice, rinsed twice with ice cold PBS, scraped from the dishes and transferred to a microfuge tube. Cells were briefly spun down and lysed by pipet resuspension in 500 μl of NP40 lysis buffer (1% NP40; 50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 5 mM EGTA; 5 mM EDTA; 15 mM $MgCl_2$; 60 mM β-glycerolphosphate; 1 mM DTT; 0.1 mM $NaVO_4$; 0.1 mM NaF; 15 mM p-nitrophenylphosphate; 1.8 μg/ml aprotinin; 1 μg/ml leupeptin; 10 μg/ml soybean trypsin inhibitor; 0.1 mM benzamidine). Lysates were kept on ice for 10 min, gently vortexed, and centrifuged for 10 min in a microfuge at 4°C. The supernatant was then filtered through a 0.45 μm low-protein binding syringe filter (Supor Acrodisc, Gelman Sciences, Ann Arbor, MI), and precleared with 15 μl protein A sepharose. Precleared lysates were immunoprecipitated using 2 μg of monoclonal 12CA5 (BAbCo, Richmond, CA) bound to 7.5 μl protein A sepharose for 1 h at 4°C. Beads were washed four times with 500 μl ice cold NP40 lysis buffer. Indirect immunofluorescence of transfected cells using anti-HA1 antibodies (12CA5) was carried out as previously described (Fornerod *et al.*, 1995).

PCR amplification

First strand cDNA was prepared from total RNA of the myeloid leukemia cell line KG-1 (Koeffler and Golde, 1978), using anti-sense primer CAN 6237A (5'-AACCA-GAAGTCTGTTGGGACAGTG-3') representing position 6237–6260 of the *can* cDNA (von Lindern *et al.*, 1992a). DNA was subsequently PCR amplified using sense primer CAN 5586SE (5'-CTTCTGGGTTCAGCTTTTGCCAAG-3') representing positions 5586–5609 and anti-sense primer CAN 6209A (5'-AAGTGGGGGCATTCTGACTCGC-3') representing positions 6209–6230 of the *can* cDNA, annealing was at 60°C. Amplified DNA derived from alternative transcripts was sequenced and cloned into

pHA1-Can using the *Hind*III and *Nru*I sites at positions 5607 and 6207 of the *can* cDNA, creating pHA1-CAN-FS1865.

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References

Bogerd HP, Fridell RA, Madore S and Cullen BR. (1995). *Cell*, **82**, 485–494.

Borrow J, Shearman AM, Stanton VP Jr, Becher R, Collins T, Williams AJ, Dube I, Katz F, Kwong YL, Morris C, Ohyashiki K, Toyama K, Rowley J and Housman DE. (1996). *Nat. Genet.*, **12**, 159–167.

Byrd DA, Sweet DJ, Pante N, Konstantinov KN, Guan T, Saphire AC, Mitchell PJ, Cooper CS, Aebi U and Gerace L. (1994). *J. Cell. Biol.*, **127**, 1515–1526.

Chi NC, Adam EJH and Adam SA. (1995). *J. Cell. Biol.*, **130**, 265–274.

Fornerod M. (1996). *Bio Techniques*, **20**, 876–877.

Fornerod M, Boer J, van Baal S, Jaeglé M, Von Lindern M, Murti KG, Davis D, Bonten J, Buijs A and Grosveld G. (1995). *Oncogene*, **10**, 1739–1748.

Fritz CC, Zapp ML and Green MR. (1995). *Nature*, **376**, 530–533.

Görlich D and Mattaj I. (1996). *Science*, **271**, 1513–1518.

Görlich D, Vogel F, Mills AD, Hartmann E and Laskey RA. (1995). *Nature*, **377**, 246–248.

Gossen M and Bujard H. (1992). *Proc. Natl. Acad. Sci. USA*, **89**, 5547–5551.

Grandi P, Emig S, Weise C, Hucho F, Pohl F, Pohl T and Hurt EC. (1995). *J. Cell. Biol.*, **130**, 1263–1273.

Henikoff S. (1984). *Gene*, **28**, 351–359.

Hurt EC. (1989). *J. Cell. Sci.*, **12**, 243–252.

Kalderon D, Roberts BL, Richardson WD and Smith AE. (1984). *Cell*, **39**, 499–509.

Kellogg DR, Kikuchi A, Fujii-Nakata T, Turck CW and Murray AW. (1995). *J. Cell. Biol.*, **130**, 661–673.

Koeffler HP and Golde DW. (1978). *Science*, **200**, 1153–1154.

Kraemer D, Wozniak RW, Blobel G and Radu A. (1994). *Proc. Natl. Acad. Sci. USA*, **91**, 1519–1523.

Kunkel TA. (1985). *Proc. Natl. Acad. Sci. USA*, **82**, 488–492.

Moroianu J, Hijikata M, Blobel G and Radu A. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 6532–6536.

Nagata K, Kawase H, Handa H, Yano K, Yamasaki M, Ishimi Y, Okuda A, Kikuchi A and Matsumoto K. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 4279–4283.

Nakamura T, Largaespada DA, Lee MP, Johnson LA, Ohyashiki K, Toyama K, Chen SJ, Willman CL, Chen IM, Feinberg AP, Jenkins NA, Copeland NG and Shaughnessy JD Jr. (1996). *Nat. Genet.*, **12**, 154–158.

Panté N, Bastos R, McMorrow I, Burke B and Aebi U. (1994). *J. Cell. Biol.*, **126**, 603–617.

Rabbitts TH. (1994). *Nature*, **372**, 143–149.

Radu A, Blobel G and Moore MS. (1995a). *Proc. Natl. Acad. Sci. USA*, **92**, 1769–1773.

Radu A, Moore MS and Blobel G. (1995b). *Cell*, **81**, 215–222.

Robbins J, Dilworth SM, Laskey RA and Dingwall C. (1988). *Cell*, **64**, 615–623.

Rout MP and Wente SR. (1994). *Trends Biochem. Sci.*, **4**, 357–363.

Rowley JD and Potter D. (1976). *Blood*, **47**, 705–721.

Siomi H and Dreyfuss G. (1995). *J. Cell. Biol.*, **129**, 551–560.

Soekarman D, Von Lindern M, Daenen S, de Jong B, Fonatsch C, Heinze B, Bartram C, Hagemeijer A and Grosveld G. (1992). *Blood*, **79**, 1–8.

Starr CM, D'Onofrio M, Park MK and Hanover JA. (1990). *J. Cell. Biol.*, **110**, 1861–1871.

Stutz F, Neville M and Rosbash M. (1995). *Cell*, **82**, 495–506.

Sundström C and Nilsson K. (1976). *Int. J. Cancer*, **171**, 565–577.

van Deursen J, Boer J, Kasper L and Grosveld G. (1996). *EMBO J.*, in press.

von Lindern M, Fornerod M, van Baal S, Jaeglé M, de Wit T, Buijs A and Grosveld G. (1992a). *Mol. Cell. Biol.*, **12**, 1687–1697.

von Lindern M, Poustka A, Lerach H and Grosveld G. (1990). *Mol. Cell. Biol.*, **10**, 4016–4026.

von Lindern M, van Baal S, Wiegant J, Raap A, Hagemeijer A and Grosveld G. (1992b). *Mol. Cell. Biol.*, **12**, 3346–3355.

Wilson IA, Niman HL, Hougten RA, Cherenson AR, Connolly ML and Lerner RA. (1984). *Cell*, **37**, 767–778.

Wimmer C, Doye V, Grandi P, Nehrbass U and Hurt EC. (1992). *EMBO J.*, **11**, 5051–5061.

6 Loss of the *DEK-CAN* fusion transcript in a child with t(6;9) acute myeloid leukemia following chemotherapy and allogeneic bone marrow transplantation

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Loss of the *DEK-CAN* fusion transcript in a child with t(6;9) acute myeloid leukemia following chemotherapy and allogeneic bone marrow transplantation

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The recurrent t(6;9)(p23;q34), found with low frequency in acute myeloid leukemia (AML) and myelodysplastic syndrome,[1,2] creates a fusion between two genes, *DEK* and *CAN*.[3] The resulting chimeric *DEK-CAN* mRNA can be detected with the reverse transcription-polymerase chain reaction (RT-PCR).[1] This technique allowed sensitive detection of residual leukemic cells after treatment in a t(6;9) patient.[4] Here, we describe for the first time the complete absence of *DEK-CAN* transcripts in post-transplantation samples from a patient who has been in continuous complete remission for more than 2 years.

In November 1993, a 5-year-old boy presented with malaise, bone pain and weight loss. Other than mild pallor, physical examination was normal. Abnormal white cells were evident on a blood smear, and complete blood counts revealed hemoglobin 4.5 g/dl, platelet count 48 000/mm^3, and white blood cell count 8300/mm^3, with 30% blasts. Other studies showed normal cerebrospinal fluid, normal coagulation values, and LDH 1363 IU (normal 420–920 IU). The bone marrow was replaced by FAB type M2 blast cells, which stained positively for myeloperoxidase and Sudan black, and negatively for α-naphthyl butyrate and chloroacetate esterase. Auer rods were not noted. Chromosome analysis revealed a clonal abnormality in 19 of 20 metaphases: 46,XY, t(6;9)(p23;q34). Immunophenotype studies of blasts were consistent with myeloid lineage: CD33$^+$ (97%), CD13$^+$ (92%) and CD15$^+$ (52%). The patient was enrolled on the AML91 protocol.[5] Hematologic remission, including normal karyotype, was achieved after two courses of 2-CDA followed by daunomycin, Ara-C, and etoposide. The patient underwent allogeneic bone marrow transplantation (BMT) from his HLA-identical brother in April 1994. The BMT conditioning regimen included total body irradiation and high-dose chemotherapy, and cyclosporin and pentoxifylline were administered to prevent graft-versus-host disease (GVHD). The patient required a short course of prednisone for probable intestinal GVHD.

Collection of bone marrow cells, cDNA preparation and PCR conditions for the evaluation of *DEK-CAN* transcripts were essentially as described,[1] using primer set 2, with the following modifications: first-strand cDNA was synthesized from 2 μg instead of 5 μg total RNA. In the PCR a new *CAN* antisense primer CEAH2 (5'-CCAGATGCTGATCCCACTCC) was used. First-round PCR was performed for 34 cycles (94°C, 1 min; 47°C, 2 min; 72°C, 2 min), the product was diluted (1:500) and 1 μl was amplified in a second round of PCR (30 cycles). Using the CEAH2 primer for two rounds of PCR sometimes resulted in background amplification of CAN. In subsequent studies on bone marrow samples from another t(6;9) patient, we found that performing a semi-nested PCR with the *CAN* antisense primer CEAH1 for the first-round, followed by CEAH2 for the second-round, gave more consistent results. One-eighth of the PCR product was resolved by electrophoresis through 1.5% agarose gels. The identity of the PCR fragments was confirmed by hybridization of Southern blots with specific oligonucleotide probes,[1] allowing the detection of one t(6;9) positive cell in 5×10^4 normal cells (not shown).

RT-PCR readily detected the *DEK-CAN* fusion transcript in the patient's bone marrow at diagnosis after 34 (not shown) and 64 cycles (Figure 1, lane 1). During chemotherapy-induced remission chimeric mRNA was only detectable after 64 cycles (Figure 1, lane 2), whereas samples taken 4 months (Figure 1, lane 3) and 25 months (Figure 1, lane 4) after BMT were negative for *DEK-CAN*. Due to low RNA yield, the sample taken 4 months after BMT had to be amplified for four additional cycles in the second-round of PCR, resulting in about a 10-fold decrease in sensitivity.

The *DEK-CAN* RT-PCR assay detects one patient cell in 5×10^4 normal cells, which is 2–20 times less sensitive than similar assays for other fusion transcripts, such as *BCR-ABL* and *AML1-ETO*.[6–8] This degree of sensitivity probably reflects low levels of *DEK-CAN* mRNA in leukemic cells.[3]

Our PCR results show that samples from a patient who has achieved complete remission following allogeneic BMT do not contain detectable amounts of *DEK-CAN* transcript. The chemotherapy alone decreased but did not completely eradicate the leukemic cells. These data support the clinical observation that t(6;9) patients respond poorly to chemotherapy, with a median survival of less than 1 year from diagnosis,[2] and that transplantation may be the most effective therapy for t(6;9) AML (our unpublished observation).

In other translocation-related leukemias, such as t(9;22) chronic myeloid leukemia and t(15;17) acute promyelocytic leukemia, the presence of fusion transcripts after treatment can be predictive of relapse, while serially negative PCR tests are associated with prolonged disease-free survival (Ref. 9 and references therein).[9,10–13] However, persistence of fusion transcripts in AML with t(8;21), APL with t(15;17) and CML with t(9;22) in prolonged complete remission has also been described, and the issue is still controversial.[14–17] In the only other t(6;9) patient who has been monitored for minimal residual disease by RT-PCR, the *DEK-CAN* mRNA was detected during and after autologous peripheral blood stem cell transplantation and the patient relapsed within 4 months.[4]

Our data show that in a child with t(6;9) AML long-term

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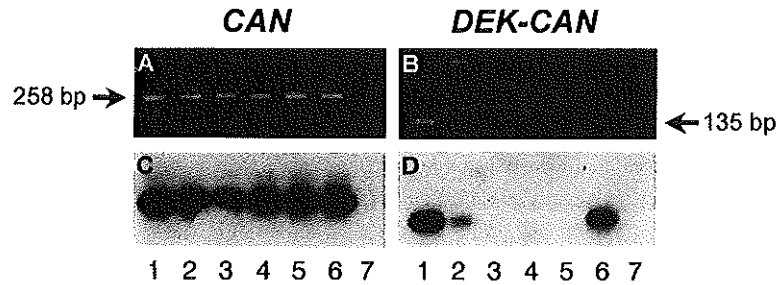

Figure 1 RT-PCR analysis of t(6;9) patient bone marrow. *CAN* and *DEK-CAN* RT-PCR products from bone marrow cells at diagnosis (lane 1), in chemotherapy-induced remission (lane 2), 4 months (lane 3) and 25 months (lane 4) after subsequent allogeneic BMT, are shown on agarose gels (A and B) and Southern blots (C and D). Cultured cells expressing normal *CAN* (HtTA-1;[18] lane 5) or transfected with a *DEK-CAN* cDNA (C4322;[19] lane 6) served as controls as did reactions to which no RNA was added (lane 7). A 258-bp *CAN* fragment was amplified in all samples containing RNA (A) and hybridized to an internal *CAN* probe (C). Primers specific for *DEK-CAN* amplified a 135-bp fragment (B) in patient samples at diagnosis (lane 1) and remission (lane 2), and in the positive control (lane 6). This band hybridized to a breakpoint-specific probe (lanes 1, 2 and 6; D).

remission after allogeneic bone marrow transplantation correlates with a negative RT-PCR for *DEK-CAN*. We believe that RT-PCR-based monitoring for residual t(6;9) cells is essential for the timely identification of patients who are at high risk of relapse.

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References

1 Soekarman D, Von Lindern M, Daenen S *et al.* The translocation (6;9)(p23;q34) shows consistent rearrangement of two genes and defines a myeloproliferative disorder with specific clinical features. *Blood* 1992; **79**: 1–8.

2 Lillington DM, MacCallum PK, Lister TA, Gibbons B. Translocation t(6;9)(p23;q34) in acute myeloid leukemia without myelodysplasia or basophilia: two cases and a review of the literature. *Leukemia* 1993; **7**: 527–531.

3 Von Lindern M, Fornerod M, van Baal S *et al.* The translocation (6;9), associated with a specific subtype of acute myeloid leukemia, results in the fusion of two genes, dek and can, and the expression of a chimeric, leukemia-specific dek-can mRNA. *Mol Cell Biol* 1992; **12**: 1687–1697.

4 Nakano H, Shimamoto Y, Suga K, Kobayashi M. Detection of minimal residual disease in a patient with acute myeloid leukemia and t(6;9) at the time of peripheral blood stem cell transplantation. *Acta Haematol* 1995; **94**: 139–141.

5 Krance R, Hurwitz C, Heslop H *et al.* AML-91 pilot study: (1) to determine the response rate to 2-CDA in previously untreated children with *de novo* AML and (2) to investigate the efficacy of autobmt by the use of NEO^R gene marking. *Blood* 1995; **86**: 433a.

6 Kawasaki ES, Clark SS, Coyne MY *et al.* Diagnosis of chronic myeloid and acute lymphocytic leukemias by detection of leukemia-specific mRNA sequences amplified *in vitro*. *Proc Natl Acad Sci USA* 1988; **85**: 5698–5702.

7 Roth MS, Antin JH, Bingham EL, Ginsburg D. Detection of Philadelphia chromosome-positive cells by the polymerase chain reaction following bone marrow transplant for chronic myelogenous leukemia. *Blood* 1989; **74**: 882–885.

8 Preudhomme C, Phillipe N, Macintyre E *et al.* Persistence of AML1/ETO fusion mRNA in t(8;21) acute myeloid leukemia (AML) in prolonged remission: is there a consensus? (letter). *Leukemia* 1996; **10**: 186–188.

9 Radich JP, Gehly G, Gooley T *et al.* Polymerase chain reaction detection of the *BCR-ABL* fusion transcript after allogeneic marrow transplantation for chronic myeloid leukemia: results and implications in 346 patients. *Blood* 1995; **85**: 2632–2638.

10 Lo Coco F, Diverio D, Pandolfi PP *et al.* Molecular evaluation of residual disease as a predictor of relapse in acute promyelocytic leukaemia. *Lancet* 1992; **340**: 1437–1438.

11 Huang W, Sun G-L, Li X-S *et al.* Acute promyelocytic leukemia: clinical relevance of two major PML-RARα isoforms and detection of minimal residual disease by retrotranscriptase/polymerase chain reaction to predict relapse. *Blood* 1993; **82**: 1264–1269.

12 Miller WH Jr, Levine K, DeBlasio A, Frankel SR, Dmitrovsky E, Warrell RP Jr. Detection of minimal residual disease in acute promyelocytic leukemia by a reverse transcription of polymerase chain reaction assay for the PML/RAR-α fusion mRNA. *Blood* 1993; **82**: 1689–1694.

13 Koller E, Karlic H, Krieger O *et al.* Early detection of minimal residual disease by reverse transcriptase polymerase chain reaction predicts relapse in acute promyelocytic leukemia. *Ann Hematol* 1995; **70**: 75–78.

14 Nucifora G, Larson RA, Rowley JD. Persistence of the 8;21 translocation in patients with acute myeloid leukemia type M2 in long-term remission. *Blood* 1993; **82**: 712–715.

15 Miyamura K, Tahara T, Tanimoto M *et al.* Long persistent bcr-abl positive transcript detected by polymerase chain reaction after marrow transplant for chronic myelogenous leukemia without clinical relapse: a study of 64 patients. *Blood* 1993; **81**: 1089–1093.

16 Maruyama F, Stass SA, Estey EH *et al.* Detection of AML1/ETO fusion transcript as a tool for diagnosis t(8;21) positive acute myelogenous leukemia. *Leukemia* 1994; **8**: 40–45.

17 Tobal K, Saunders MJ, Grey MR, Liu Yin JA. Persistence of RARα-PML fusion mRNA detected by reverse transcriptase polymerase chain reaction in patients in long-term remission of acute promyelocytic leukaemia. *Br J Haematol* 1995; **90**: 615–618.

18 Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracyclin-responsive promoters. *Proc Natl Acad Sci USA* 1992; **89**: 5547–5551.

19 Fornerod M, Boer J, van Baal S *et al.* Relocation of the carboxyterminal part of CAN from the nuclear envelope to the nucleus as a result of leukemia-specific chromosome rearrangements. *Oncogene* 1995; **10**: 1739–1748.

7 Summary and Discussion

Summary

The molecular cloning of recurring chromosomal translocation breakpoints has provided a starting point from which the pathogenesis of human leukemias can be studied. Translocation (6;9)(p23;q34) is found with low frequency in acute myeloid leukemia and myelodysplastic syndrome. The translocation breakpoints occur in the *DEK* gene on chromosome 6 and in the *CAN* gene on chromosome 9, generating a *DEK-CAN* fusion gene. In one case of acute undifferentiated leukemia, the same part of *CAN* is fused to *SET*, on chromosome 9q34. The *CAN* gene encodes a nucleoporin, a component of the nuclear pore complex (NPC). NPCs are composed of multiple copies of about 100 different proteins and form pores in the nuclear membrane through which active transport of macromolecules between the cytoplasm and the nucleus occurs. A number of these proteins, including CAN, contains nucleoporin-specific FG peptide repeat motifs (Fornerod et al., 1995; Kraemer et al., 1994). DEK and SET are both nuclear, and so are the two fusion proteins (Fornerod et al., 1995). The aim of these studies was to gain insight into the functions of CAN and the role of CAN-derived fusion proteins in leukemogenesis.

To study the normal functions of CAN, we first disrupted the *CAN* gene by homologous recombination in mouse embryonic stem (ES) cells. ES cells lacking CAN were not viable, suggesting that CAN is essential for cell growth or survival. Accordingly, embryos homozygous for the disrupted *CAN* allele died *in utero* between 4.0 and 4.5 days postcoitum, following the depletion of their maternal CAN. The CAN-depleted embryo cells arrested in the G_2 phase of the cell cycle. We examined if transport of substrates through the nuclear pore was affected by CAN depletion and found that the import of nuclear localization sequence (NLS)-containing proteins into the nucleus was impaired. Moreover, the export of polyadenylated RNA from the nucleus to the cytoplasm was blocked. Therefore, we conclude that CAN is essential for cell cycle progression and NPC function *in vivo* (Chapter 2).

In the search for clues about the function of CAN in nucleocytoplasmic transport and the CAN-derived fusion proteins in leukemia, our group identified proteins that interact with CAN. Two proteins were found that specifically coimmunoprecipitated with CAN, one of 88 kDa and the other of 112 kDa. Mutational analysis of CAN demonstrated that the 88 kDa protein associated with the central region of CAN, which contains several protein-protein interaction domains. Almost the same region of CAN was responsible for its localization at the NPC. The fusion of CAN to DEK or SET occurs in this region, disrupting both NPC association and binding of the 88 kDa protein. The 112 kDa co-precipitating protein bound to the C-terminal FG repeat region of CAN and also interacted with DEK-CAN and SET-CAN. An overlapping region caused nuclear localization of CAN mutants that had lost the NPC binding region

(Chapter 5). Recently, the 88 kDa protein was cloned and characterized as a novel NPC component and therefore named NUP88. The 112 kDa CAN-interacting protein was identified as the human homolog of yeast CRM1, a protein known to be required for maintenance of correct chromosome structure. This human CRM1 (hCRM1) localizes to the nucleus and to the NPC, and has characteristics of a transport factor (Fornerod et al., 1997).

Next, we investigated the effect of CAN and DEK-CAN expression on the growth and differentiation of myeloid precursor U937 cells. Since CAN and DEK-CAN had proven to be cytotoxic upon overexpression, we used an inducible expression system. We found that DEK-CAN expression did not affect terminal myeloid differentiation of U937 cells. Possibly, DEK-CAN affects a differentiation step of earlier myeloid precursor cells in t(6;9) acute myeloid leukemia patients. Alternatively, since myeloid cells from t(6;9) patients are partially inhibited in their differentiation pathways but are not totally blocked, DEK-CAN could affect proliferation rather than differentiation. CAN-overexpressing U937 cells exited the cell cycle and arrested in G_0, developed a block in mRNA export, and ultimately died by apoptosis. Interestingly, the overexpressed CAN protein accumulated in the nucleus where it colocalized with importin β and hCRM1, depleting both factors from the nuclear envelope. Expression of CAN's C-terminal FG repeat region alone, comprising the binding region for hCRM1, also resulted in colocalization with hCRM1 in the nucleoplasm and was sufficient to inhibit proliferation and to induce cell death. These data suggest that sequestering of hCRM1 by an excess of CAN inhibits an essential transport function of hCRM1 (Chapter 3).

Immunoelectron microscopic studies designed to study the localization of CAN within the NPC, showed that in moderately overexpressing HeLa cells both the cytoplasmic and nucleoplasmic fibrils of the NPC contain specific binding sites for CAN, or for its interacting proteins. This finding makes it conceivable that CAN functions in nucleocytoplasmic transport at both faces of the NPC (Chapter 4).

The chimeric *DEK-CAN* messenger RNA is a target for diagnosis via complementary DNA preparation followed by amplification of the breakpoint region in a polymerase chain reaction. We extended this method to the sensitive detection of minimal amounts of leukemic cells after treatment of a t(6;9) acute myeloid leukemia patient. The absence of the chimeric mRNA in the bone marrow after allogeneic bone marrow transplantation correlated with disease-free survival, suggesting that this parameter can be used as a prognostic factor (Chapter 6).

These studies show that CAN is essential for nucleocytoplasmic transport. Moreover, CAN could play an indirect role in the progression of the cell cycle. It is less clear how an NPC component could contribute to the development of leukemia upon fusion to a nuclear protein. Possibly, the CAN-derived fusion proteins interfere with the transport function of CAN. Alternatively, the addition of CAN sequences to DEK or SET could deregulate the functions of these proteins in transcription or replication of DNA.

CAN is essential for nucleocytoplasmic transport and cell cycle progression

CAN is a nuclear pore complex (NPC) component and studies described in this thesis demonstrate that CAN has an essential role in nucleocytoplasmic trafficking. Both absence and overexpression of CAN inhibit transport without causing structural perturbations of the nuclear envelope or NPC. CAN-depleted mouse embryo cells accumulate polyadenylated RNA in their nuclei and concomitantly show decreased protein import. A similar block in mRNA export is detected in U937 cells that overexpress CAN. Thus, overexpression of CAN interferes with some of CAN's normal functions in a dominant-negative way. CAN function is also important for the progression of the cell cycle, since knock-out embryo cells arrest in G_2 and overexpressing myeloid cells in G_0 (Chapters 2 and 3).

Both CAN overexpression and depletion have consequences for the subcellular localization of a number of CAN-interacting proteins, which could in part be responsible for the phenotypes. NUP88 and hCRM1 are CAN-interacting proteins that were identified by coimmunoprecipitation techniques (Chapter 5 and Fornerod et al., 1997). Importin β, also called p97, is a transport factor that shares a region of homology with hCRM1 and other putative NPC-interacting transport factors that belong to the newly identified CRIME family (Fornerod et al., 1997). Importin β interacts with CAN and several other peptide repeat-containing nucleoporins in an *in vitro* ligand blot assay (Moroianu et al., 1995; Radu et al., 1995a). In embryonic cells that lack CAN, NUP88 is no longer detectable at the NPC, thus CAN depletion indirectly causes a defect in NUP88 function. hCRM1 localizes very prominently to the nucleoli of CAN-depleted embryos, which is not observed in wild-type embryos. This suggests that the nucleolus is part of normal hCRM1 routing, which may be disturbed in cells lacking CAN. However, hCRM1 remains present at the NPC in CAN knock-out cells, indicating that hCRM1 binds to other nucleoporins in addition to CAN (Fornerod et al., 1997). Similarly, importin β still interacts with NPCs that lack CAN, presumably contacting other repeat-containing nucleoporins (Chapter 2). Bidirectional transport through the NPC may require ligand-bound transport receptors to dock and undock along an array of repeat-containing nucleoporins across the NPC (Moroianu et al., 1995). CAN depletion could disrupt this process, thereby partly or completely blocking transport of certain substrates.

CAN-overexpressing U937 cells accumulate overexpressed CAN protein in the nucleus. Here, it colocalizes with hCRM1 and importin β, causing depletion of both these factors from the NPC and potentially disturbing their transport functions. The cell cycle arrest in G_0 coincides with cessation of nuclear protein import. Unfortunately, U937 control cells arrested in G_1/G_0 after reaching their maximal cell density do not

import detectable amounts of import substrate either. Therefore, an additional effect on import of importin β or hCRM1 sequestering in the nucleus could not be determined. A similar growth arrest is detected in U937 cells that only overexpress the hCRM1-binding domain of CAN. This nuclear mutant colocalizes with endogenous hCRM1 but does not disturb importin β localization. Only cells overexpressing full-length CAN develop a defect in polyadenylated RNA export. These were also the only cells to sequester importin β in their nuclei. The nuclear RNA accumulation could be secondary to importin β inactivation, since the partner of importin β in the heteodimeric import receptor complex, importin α, also functions in the export of capped RNA polymerase II transcripts (Görlich et al., 1996). In addition to hCRM1 and importin β, other CRIME family proteins could also be inhibited in their function upon CAN depletion or overexpression. The discovery of this family suggests a differentiation among transport receptors for specific types of substrates.

Moderate overexpression of CAN in HeLa cells resulted in an increase of CAN at the nuclear envelope, where it localized to both the cytoplasmic and nucleoplasmic aspects of the NPC (Chapter 4). In previous immunolocalization studies CAN is detected only at the cytoplasmic face of NPCs of normal HeLa cells and *Xenopus* oocytes (Kraemer et al., 1994; Panté et al., 1994). Our data indicate that not only the cytoplasmic fibrils, but also the nucleoplasmic structures of the NPC contain specific binding sites for either CAN or for its interacting proteins, NUP88 and hCRM1. Thus, it is conceivable that CAN functions in nucleocytoplasmic transport at both faces of the NPC. Clearly, at endogenous expression levels in HeLa cells not all NPC-binding sites for CAN are occupied. Although all examined cell types express CAN, the level of expression varies. Relatively high levels of CAN are expressed in HeLa epithelial cells, NIH 3T3 fibroblasts, and embryonal trophectoderm cells, whereas CAN levels are low in U937 myeloid precursors, ES cells and inner cell mass cells of 4.5-day-old mouse embryos (Chapter 2 and unpublished observations). Possibly, the expression level of CAN may reflect the nucleocytoplasmic transport activity of specific CAN substrates.

hCRM1 binding

Colocalization proved to be a powerful technique to further map the interaction between the hCRM1-binding domain of CAN and hCRM1. CAN mutants with a C-terminal or internal deletion of the hCRM1-binding domain no longer coimmunoprecipitated hCRM1, but still localized mainly to the nucleoplasm (Chapter 5). These mutants colocalized with endogenous hCRM1 in overexpressing U937 cells, thus it seems likely that they bind hCRM1, albeit with a lower affinity, and are transported to the nucleus in a complex with hCRM1 (Chapter 3). The nuclear accumulation of full-length CAN upon

overexpression in U937 cells could also be explained by binding to hCRM1 (Chapter 3). Colocalization of CAN and hCRM1 in cytoplasmic structures suggests that complex formation already occurs in the cytoplasm. When all NPC binding sites for CAN are occupied, the complex could spill over to the nucleus. Since hCRM1 has a half-life of approximately 24 hours (Fornerod et al., 1997), it is unlikely that the hCRM1 observed in the cytoplasm of CAN-overexpressing cells is only newly synthesized hCRM1. Instead, these data suggest that hCRM1 travels from the nucleus to the cytoplasm, in addition to its release from the NPC into the nucleoplasm. In normal HeLa cells, a small amount of hCRM1 is present in cytoplasmic annulate lamellea (Fornerod et al., 1997).

To ensure directed transport across the nuclear pore complex, transport factors must somehow "sense" a cytoplasmic or nucleoplasmic environment. It is possible that the conditions in the nucleoplasm are not favorable for dissociation of hCRM1 and CAN, resulting in the nuclear accumulation of hCRM1/CAN complexes. Depletion of hCRM1 from the NPC correlates with lethality, indicating that hCRM1 function is essential for cell survival. The localization of hCRM1 is the mirror image of that of importin β, suggesting that the direction of transport is from the nucleus to the cytoplasm (Fornerod et al., 1997). As yet, we do not have clues regarding the transport substrate of hCRM1. All we can say with certainty is that it is not essential for mRNA export. The hCRM1-binding domain overexpressing U937 cells should provide a good model to investigate potential substrates.

Importin β interaction

The colocalization of overexpressed CAN with endogenous importin β suggests that these two proteins interact *in vivo*. Notably, importin β did not colocalize with mutants expressing only the C-terminal repeat-region of CAN, whereas corresponding repeat-rich regions in both the vertebrate NUP98 and yeast NUP159 were mapped to bind importin β (Kraemer et al., 1995; Radu et al., 1995b). A CAN mutant extending from the t(6;9) breakpoint to the C-terminus colocalized with importin β in cytoplasmic speckles. This mutant contains part of the central NPC-association region of CAN, suggesting that CAN's central region is required for importin β interaction, either directly or via binding of another protein. We could not determine if this region is also sufficient for importin β binding, since the central region of CAN localizes only to the nuclear envelope, which is a natural location of importin β. Although it has been speculated that importin β directly binds to FG repeat regions, our data do not support this model. It is possible that, in contrast to *in vitro* binding, the *in vivo* interaction between importin β and CAN's nucleoporin-specific repeats requires the NPC-binding domain of CAN. Recent binding studies show that importin β has at least two non-overlapping sites of interaction with

the NPC. One of these sites is located at the N-terminus and includes the CRIME domain. The N-terminus of importin β is also involved in RanGTP binding (Kutay et al., 1997).

CAN-derived fusion proteins

What is the role of DEK-CAN and SET-CAN in acute leukemia? The transformation potential of these fusion proteins is not easily demonstrated in *in vitro* or *in vivo* experimental systems (unpublished data), therefore we have not been able to map functional domains within the fusion proteins. However, comparing the CAN-derived fusion proteins to the structurally similar NUP98-HOXA9 fusion protein could give useful insights into their function (Figure 1). DEK-CAN, SET-CAN (Fornerod et al., 1995) and NUP98-HOXA9 (J. van Deursen, personal communication) are all nuclear proteins. I will discuss the possibilities of these fusion proteins to interfere with nucleocytoplasmic transport or transcription and suggest some future directions.

Nucleoporin repeat-containing fusion proteins in nucleocytoplasmic transport

The intriguing finding of two FG repeat-containing nucleoporins, CAN and NUP98, as part of leukemia-associated fusion proteins, opens a new field of research into the role of nucleocytoplasmic trafficking in oncogenesis. Abnormal nuclear pore function could result from the haploinsufficiency of NUP98 and CAN. Alternatively, NUP98-HOXA9

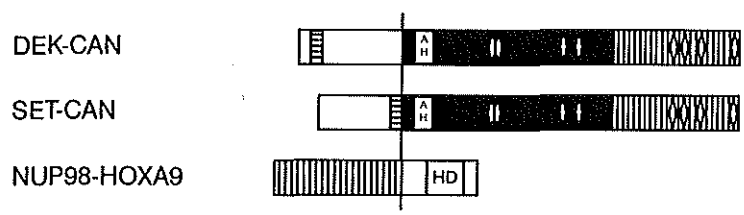

Figure 1. Nucleoporin-derived oncoproteins

Structural similarities between three leukemia-associated fusion proteins that contain nucleoporin-specific peptide repeats, DEK-CAN, SET-CAN, and NUP98-HOXA9. Diamonds, FXF repeats; Vertical bars, FG repeats; AH, amphipathic helix; Horizontal bars, acidic regions; HD, homeodomain.

and the CAN-derived fusion proteins could interfere with the transport functions of their wild-type nucleoporin counterparts. The nucleoporin-derived fusion proteins are nuclear and could cause a redistribution of transport factors towards the nucleoplasm. DEK-CAN and SET-CAN tightly bind hCRM1, and could relocate part of the endogenous hCRM1 to the nucleus. These fusion proteins are expressed at such low levels in leukemic cells (Chapter 6 and Von Lindern et al., 1992), that a total depletion of hCRM1 from the NPC is not to be expected. NUP98-HOXA9 contains the majority of NUP98's FG peptide repeats and would therefore be expected to retain the ability to bind importin β, potentially with an adverse effect on nucleocytoplasmic transport (Borrow et al., 1996). Other, as yet unidentified transport factors may also bind to the nucleoporin moieties of these fusion proteins, resulting in altered transport that could interfere with the growth or differentiation of hematopoietic precursor cells.

Fused transcription factors containing nucleoporin repeats

Another possibility is that the nucleoporin-derived fusion proteins function as altered DNA-binding factors. In this respect several parallels between the HOXA9, DEK and SET proteins are interesting. All these proteins are nuclear and interact with DNA. HOXA9 is a sequence-specific transcription factor that belongs to the family of homeodomain proteins, which has been implicated in normal differentiation and development as well as in cancer (Chapter 1). Its DNA-binding homeodomain and flanking sequences that could be important for interactions with related proteins are present in the NUP98-HOXA9 fusion protein (Borrow et al., 1996; Nakamura et al., 1996).

DEK was recently discovered to be a sequence-specific DNA-binding protein, and DEK binding sites were identified in the myeloperoxidase promoter and the HIV-2 long terminal repeat (Fu et al., 1997). Since DEK has no significant homology to other proteins, it is not clear which domain interacts with DNA. Earlier studies into the transactivation potential of DEK, fused to the DNA-binding domain of OCT6, revealed that DEK has very low transactivation activity on a chloramphenicol acetyl transferase (CAT) reporter gene preceded by six OCT6 consensus binding sites (unpublished data). However, it is possible that DEK needs to interact with other transcription factors bound to a promoter or enhancer to reach its full transactivation potential.

Finally, SET has been implicated in several nuclear processes (Chapter 1). The most exciting finding is that SET binds to MLL, a large AT hook-containing protein that is frequently translocated in acute leukemias. The SET interaction domain within MLL is located between the AT hooks (Adler and Tkachuk, 1996). A complex of MLL, SET, and possibly other factors, could bind to AT-rich sequences in the minor groove of DNA via the AT hooks of MLL and facilitate transcription by altering chromatin structure. In

this scenario, SET would be an architectural factor, rather than a classical transcription factor. The homology between SET and NAP-1, and SET's TAF-I activity are in agreement with a regulatory role in chromatin structure (Kellogg et al., 1995; Nagata et al., 1995). Both SET-CAN and MLL-derived fusion proteins may interfere with critical growth or differentiation pathways in early pluripotential hematopoietic cells. Since almost the entire DEK and SET polypeptides are present in DEK-CAN and SET-CAN, respectively, domains that directly (DEK), or indirectly (SET) interact with DNA may very well be present in the fusion proteins.

Several ways can be envisioned by which nucleoporin-derived sequences could interfere with the transcriptional regulation of DNA-binding factors. First, the nucleoporin repeats could harbor a transcriptional activation domain. In the OCT6-fusion reporter system described above, a transcriptional activation domain similar in strength to wild-type OCT6 was mapped to the repeat-containing region of CAN (unpublished results). In respect to SET-CAN, it is of interest that several of the fusion partners of MLL may be transcriptional regulators (Chapter 1). It is also possible that not CAN itself, but a protein interacting with its repeat sequence, has transactivation activity.

Second, the addition of nucleoporin repeats could alter the DNA binding activity or specificity of sequence-specific DNA-binding factors. The identification of the DNA binding site for DEK opens the way to investigate the effects of DEK-CAN on DNA-binding and transcriptional activation of DEK target genes.

A third mechanism by which these fusion proteins could interfere with transcription, is through binding of other factors to their transcription factor moiety. In order to understand NUP98-HOXA9 function, a comparison with E2A-PBX1 could be useful: the domain of PBX1, involved in cooperative DNA binding with a subset of HOX proteins, is required for transformation by E2A-PBX1 (Chapter 1). This region, and possibly a similar protein-protein interaction domain in HOXA9, could heterodimerize with other transcription factors which direct the fusion proteins to appropriate target genes. It is also possible that this region of the fusion proteins mediates the formation of non-DNA-binding complexes that sequester tumor-suppressing factors. A search for DEK-interacting proteins could identify factors that cooperate with DEK in the transcriptional regulation of target genes, which could be deregulated in t(6;9) leukemic cells. SET interacts with MLL and could by required for MLL function, for instance by facilitating its binding to DNA. All MLL-derived fusion proteins contain the N-terminal AT hooks, which makes it likely that they still interact with SET. The compelling question arises whether SET-CAN also interacts with MLL, and how that would alter the transcriptional activity of MLL. The first part of this question could be answered by coimmunoprecipitation studies employing cells that co-express an epitope-tagged version of MLL (Caslini et al., 1996) and SET-CAN.

Concluding remarks

The overview of leukemia-specific fusion proteins in Chapter 1 suggests that in most cases both fusion partners contribute to the development of leukemia, and the available data suggest that the effectiveness of oncogenic fusion proteins depends on this dual action. Therefore, probably more than one of the mechanisms described above is at work in leukemic cells expressing DEK-CAN or SET-CAN. New insights into the functions of these fusion proteins can be expected from the study of DEK and SET. In addition, further characterization of the CAN-interacting proteins may link CAN to cellular pathways that could be deregulated in leukemic cells and improve our understanding of the mechanism of nucleocytoplasmic transport.

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References

Adler, H., and Tkachuk, D. (1996). Leukemic HRX fusion proteins contain novel protein-protein/DNA interaction domains that bind another leukemic factor, the set protein. Blood *88*, 549a.

Borrow, J., Shearman, A. M., Stanton, V. P., Jr., Becher, R., Collins, T., Williams, A. J., Dube, I., Katz, F., Kwong, Y. L., Morris, C., Ohyashiki, K., Toyama, K., Rowley, J., and Housman, D. E. (1996). The t(7;11)(p15;p15) translocation in acute myeloid leukaemia fuses the genes for nucleoporin NUP98 and class I homeoprotein HOXA9. Nat Genet *12*, 159-167.

Caslini, C., Murti, K. G., Ashmun, R., Domer, P. H., Korsmeyer, S. J., Boer, J., Grosveld, G., and Look, A. T. (1996). Subcellular localization and cell cycle effects of the MLL-AF4 fusion oncoprotein. Blood *88*, 557a.

Fornerod, M., Boer, J., Van Baal, S., Jaeglé, M., Von Lindern, M., Murti, K. G., Davis, D., Bonten, J., Buijs, A., and Grosveld, G. (1995). Relocation of the carboxyterminal part of CAN from the nuclear envelope to the nucleus as a result of leukemia-specific chromosome rearrangements. Oncogene *10*, 1739-1748.

Fornerod, M., Van Deursen, J., Van Baal, S., Reynolds, A., Davis, D., Murti, K. G., and Grosveld, G. (1997). The human homologue of yeast CRM1 is in a dynamic subcomplex with CAN/Nup214 and a novel nuclear pore component Nup88. EMBO J *16*, 807-816.

Fu, G., Grosveld, G., and Markovitz, D. (1997). DEK, an autoantigen involved in a chromosomal translocation in acute myelogenous leukemia, binds to the human immunodeficiency virus type 2 enhancer. Proc Natl Acad Sci USA *94*, 1811-1815.

Görlich, D., Kraft, R., Kostka, S., Vogel, F., Hartmann, E., Laskey, R. A., Mattaj, I. W., and Izaurralde, E. (1996). Importin provides a link between nuclear protein import and U snRNA export. Cell *87*, 21-32.

Kellogg, D. R., Kikuchi, A., Fujii-Nakata, T., Turck, C. W., and Murray, A. W. (1995). Members of the NAP/SET family of proteins interact specifically with B-type cyclins. J Cell Biol *130*, 661-673.

Kraemer, D., Wozniak, R. W., Blobel, G., and Radu, A. (1994). The human can protein, a putative oncogene product associated with myeloid leukemogenesis, is a nuclear pore complex protein that faces the cytoplasm. Proc Natl Acad Sci USA *91*, 1519-1523.

Kraemer, D. M., Strambio-de-Castillia, C., Blobel, G., and Rout, M. P. (1995). The essential yeast nucleoporin NUP159 is located on the cytoplasmic side of the nuclear pore complex and serves in karyopherin-mediated binding of transport substrate. J Biol Chem *270*, 19017-19021.

Kutay, U., Izaurralde, E., Bischoff, F. R., Mattaj, I. W., and Görlich, D. (1997). Dominant-negative mutants of importin-β block multiple pathways of import and export through the nuclear pore complex. EMBO J *16*, 1153-1163.

Moroianu, J., Hijikata, M., Blobel, G., and Radu, A. (1995). Mammalian karyopherin α1β and α2β heterodimers: α1 or α2 subunit binds nuclear localization signal and β subunit interacts with peptide repeat-containing nucleoporins. Proc Natl Acad Sci USA *92*, 6532-6536.

Nagata, K., Kawase, H., Handa, H., Yano, K., Yamasaki, M., Ishimi, Y., Okuda, A., Kikuchi, A., and Matsumoto, K. (1995). Replication factor encoded by a putative oncogene, *set*, associated with myeloid leukemogenesis. Proc Natl Acad Sci USA *92*, 4279-4283.

Nakamura, T., Largaespada, D. A., Lee, M. P., Johnson, L. A., Ohyashiki, K., Toyama, K., Chen, S. J., Willman, C. L., Chen, I. M., Feinberg, A. P., Jenkins, N. A., Copeland, N. G., and Shaughnessy, J. D., Jr. (1996). Fusion of the nucleoporin gene NUP98 to HOXA9 by the chromosome translocation t(7;11)(p15;p15) in human myeloid leukaemia. Nat Genet *12*, 154-158.

Pante, N., Bastos, R., McMorrow, I., Burke, B., and Aebi, U. (1994). Interactions and three-dimensional localization of a group of nuclear pore complex proteins. J Cell Biol *126*, 603-617.

Radu, A., Blobel, G., and Moore, M. S. (1995a). Identification of a protein complex that is required for nuclear protein import and mediates docking of import substrate to distinct nucleoporins. Proc Natl Acad Sci USA *92*, 1769-1773.

Radu, A., Moore, M. S., and Blobel, G. (1995b). The peptide repeat domain of nucleoporin Nup98 functions as a docking site in transport across the nuclear pore complex. Cell *81*, 215-222.

Von Lindern, M., Van Baal, S., Wiegant, J., Raap, A., Hagemeijer, A., and Grosveld, G. (1992). can, a putative oncogene associated with myeloid leukemogenesis, can be activated by fusion of its 3' half to different genes: characterization of the set gene. Mol Cell Biol *12*, 3346-3355.

Samenvatting

Functionele karakterisatie van de nucleoporine CAN en CAN-afgeleide leukemie-specifieke fusie-eiwitten

Chromosomale translokaties zijn microscopisch zichtbare genetische afwijkingen die vaak geassocieerd zijn met specifieke tumoren. De karakterisatie van de genen op de chromosomale breukpunten kan inzicht geven in de processen die een normale cel transformeren tot een tumorcel. Translokatie (6;9) komt met lage frequentie voor bij acute myeloïde leukemie en myelodysplastisch syndroom. De translokatie-breukpunten treden op in het *DEK* gen op chromosoom 6 en het *CAN* gen op chromosoom 9, en leiden tot de vorming van een *DEK-CAN* fusie-gen, dat codeert voor een DEK-CAN fusie-eiwit. In een geval van acute ongedifferentieerde leukemie is hetzelfde deel van *CAN* gefuseerd met *SET*, op chromosoom 9, en wordt waarschijnlijk een SET-CAN eiwit geproduceerd. Het *CAN* gen codeert voor een nucleoporine, een component van het nucleaire porie comlex (NPC). NPC's zijn opgebouwd uit ongeveer 100 verschillende eiwitten en vormen poriën in de kernmembraan waardoor actief transport van macromoleculen tussen het cytoplasma en de kern plaatsvindt. Een aantal van deze eiwitten, waaronder CAN, bevat een gerepeteerd aminozuurmotief en speelt waarschijnlijk een rol in het transport van substraten door de kernporie. De DEK en SET eiwitten zijn gelokaliseerd in de kern, net als de DEK-CAN en SET-CAN fusie-eiwitten. Het doel van dit onderzoek was om inzicht te verkrijgen in de functies van CAN en de rol van de CAN-afgeleide fusie-eiwitten in het ontstaan van leukemie.

Om de normale functies van CAN te onderzoeken, inactiveerden we het *CAN* gen in embryonale stamcellen (ES cellen) van de muis met behulp van homologe recombinatie. ES cellen zonder CAN waren niet levensvatbaar. Dit wijst erop dat CAN van essentieel belang is voor celgroei of overleving. Muizenembryo's die homozygoot waren voor het verstoorde *CAN* gen stierven tussen 4,0 en 4,5 dag na de bevruchting, wanneer hun maternale CAN voorraad was uitgeput. De CAN-gedepleteerde embryocellen stopten met groeien en blokkeerden in de G_2 fase van de celcyclus. We bestudeerden of het transport van substraten door de kernporie werd beïnvloed door CAN-depletie, en vonden dat de import van kerneiwitten was verminderd. Bovendien lag de export van gepolyadenyleerde RNA moleculen van de kern naar het cytoplasma stil. Onze conclusie uit deze experimenten is dat CAN essentieel is voor de voortgang van de celcyclus en de functie van het NPC *in vivo*.

Op zoek naar aanknopingspunten voor de functies van CAN, identificeerde onze groep eiwitten die een interactie aangaan met CAN in de cel. We vonden twee eiwitten die specifiek binden aan CAN, een van 88 kDa en een van 112 kDa. Mutatie-analyse van CAN liet zien dat het 88 kDa eiwit associeerde met het middelste gedeelte van CAN, dat een aantal eiwit-eiwit interactiemotieven bevat. Bijna hetzelfde gedeelte was verantwoordelijk voor de lokalisatie van CAN aan het NPC. De fusie van CAN aan DEK

of SET treedt op in dit gebied, waarbij zowel de binding aan het NPC als de interactie met het 88 kDa eiwit verloren gaat. Het 112 kDa eiwit bond aan het C-terminale gerepeteerde gedeelte van CAN en associeerde ook met DEK-CAN en SET-CAN. Een overlappend gebied veroorzaakte nucleaire lokalisatie van CAN mutanten die de NPC bindingsplaats misten. Recentelijk is het 88 kDa eiwit geïsoleerd en gekarakteriseerd als een nieuwe component van het NPC, NUP88 genaamd. Het CAN-bindende eiwit van 112 kDa werd geïdentificeerd als de humane homoloog van gist CRM1, een eiwit dat nodig is voor instandhouding van een correcte chromosomale structuur. Dit humane CRM1 (hCRM1) lokaliseert in de kern en aan het NPC, en heeft eigenschappen van een transportfactor.

Vervolgens bestudeerden we de gevolgen van CAN en DEK-CAN expressie op de groei en differentiatie van U937 myeloïde voorlopercellen. Aangezien gebleken was dat overexpressie van deze eiwitten cytotoxisch is, gebruikten we een induceerbaar expressiesysteem. We vonden dat DEK-CAN expressie geen invloed heeft op de terminale myeloïde differentiatie van U937 cellen. Het is mogelijk dat DEK-CAN een differentiatiestap van vroegere myeloïde voorlopercellen beïnvloedt in t(6;9) patiënten. Aangezien de patiëntencellen gedeeltelijk in hun differentiatie geremd zijn, maar niet volledig geblokkeerd, zou DEK-CAN ook de proliferatie kunnen beïnvloeden in plaats van de differentiatie. De overexpressie van CAN remde de groei van U937 cellen, ze verlieten de celcyclus en accumuleerden in G_0. CAN-overexpresserende cellen ontwikkelden een defect in mRNA export en stierven uiteindelijk apoptotisch. Het overgeëxpresseerde CAN eiwit hoopte zich op in de kern waar het colokaliseerde met hCRM1 en importin β, een cytoplasmatische transportfactor die bindt aan het NPC via de gerepeteerde gebieden in nucleoporines. Beide factoren werden hierdoor weggevangen uit het NPC. Expressie van CAN's gerepeteerde C-terminus alleen, de bindingsplaats voor hCRM1, resulteerde ook in de accumulatie van hCRM1 in het nucleoplasma en was voldoende om de celgroei te remmen en celdood te induceren. Deze resultaten suggereren dat de binding van hCRM1 door een overmaat CAN een essentiële transportfunctie van hCRM1 remt.

Om de lokalisatie van CAN binnen het NPC in kaart te brengen, maakten we gebruik van immuno-elektronenmicroscopische technieken. Bestudering van HeLa cellen die een gematigde hoeveelheid CAN overexpresseerden, liet zien dat zowel de cytoplasmatische als de nucleoplasmatische fibrillen van het NPC specifieke bindingsplaatsen bevatten voor CAN, of voor CAN-bindende eiwitten. Deze waarneming maakt het voorstelbaar dat CAN functioneert in nucleocytoplasmatisch transport aan beide zijden van het NPC.

Het chimere DEK-CAN mRNA vormt een aangrijpingspunt voor de diagnostiek van t(6;9), aangezien het breukpuntgebied geamplificeerd kan worden met behulp van de polymerase kettingreactie. Wij breidden deze methode uit voor de gevoelige detectie van minimale hoeveelheden leukemische cellen na behandeling van t(6;9) acute

myeloïde leukemiepatiënten. De afwezigheid van het chimere mRNA in het beenmerg van een t(6;9) patiënt na allogene beenmergtransplantatie correleerde met ziekte-vrije overleving. Dit wijst erop dat de polymerase kettingreactie gebruikt kan worden als prognostische factor in de behandeling van t(6;9) patiënten.

De beschreven studies hebben ons geleerd dat CAN van cruciaal belang is voor de import van kerneiwitten en de export van mRNA door de kernporie. Indirect zou CAN door regulatie van nucleocytoplasmatisch transport de voortgang van de celcyclus kunnen beïnvloeden. Minder duidelijk is hoe een component van het NPC kan bijdragen aan het ontstaan van leukemie, wanneer het gefuseerd is aan een nucleair eiwit. Het is mogelijk dat de CAN-afgeleide fusie-eiwitten de transportfunctie van CAN ontregelen. Ook zou de toevoeging van CAN sequenties aan DEK en SET gevolgen kunnen hebben voor functies van deze eiwitten in de transcriptie of replicatie van DNA.

Abbreviations

ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
APC	allophycocyanin
APL	acute promyelocytic leukemia
AUL	acute undifferentiated leukemia
C	carboxy
CML	chronic myeloid leukemia
ES	embryonic stem
FG	phenylalanine-glycine (one letter abbreviation of amino acids)
kDa	kilodalton
MDS	myelodysplastic syndrome
N	amino
NLS	nuclear localization sequence
NPC	nuclear pore complex
NUP	nuclear pore complex component or nucleoporin
NUP214	nucleoporin of 214 kDa, also called CAN
RA	retinoic acid
RNP	ribonucleoprotein
RT-PCR	reverse transcription-polymerase chain reaction
tt	HA1-epitope tagged

Epilogue

Many have contributed in many ways to this thesis. Thank you all!

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Curriculum Vitae

Judith Mary Boer was born on August 18, 1967, in Haarlem, the Netherlands. She graduated from the Mendel College with a VWO-B diploma in 1985, and that same year she enrolled in the study Biomedical Sciences at the Medical Faculty of Leiden University, the Netherlands. Her main subjects concerned research projects in molecular biology at the Department of Medical Biochemistry of the Medical Faculty, Leiden University, supervised by Drs Arthur Osterop and Anton Maassen, and the Department of Eukaryotic Gene Expression of the National Institute for Medical Research in London (Great Britain), supervised by Drs Siu-Pok Yee and Peter Rigby. In 1988 she received a price from the Medical Faculty for a short research project and in 1990 she graduated *cum laude*. From 1991 to 1993 the author worked as a temporary research assistant (AIO) at the Department of Genetics and Cell Biology of the Faculty of Medicine and Health Sciences, Erasmus University Rotterdam, supervised by Dr Gerard Grosveld and Prof.dr Dirk Bootsma. In 1993 she continued her graduate research with Dr Gerard Grosveld at the newly started Department of Genetics of St. Jude Children's Research Hospital in Memphis (USA). Per May 1, 1997, Judith Boer works as a post-doctoral fellow at the Department of Molecular Genome Analysis of the German Cancer Research Institute in Heidelberg, Germany with Dr Annemarie Poustka.

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Publications

Fornerod, M., **Boer, J.**, Van Baal, S., Jaeglé, M., Von Lindern, M., Murti, K.G., Davis, D., Bonten, J., Buijs, A., and Grosveld, G. (1995). Relocation of the carboxyterminal part of CAN from the nuclear envelope to the nucleus as a result of leukemia-specific chromosome rearrangements. Oncogene *10*, 1739-1748.

Fornerod, M., **Boer, J.**, Van Baal, S., Morreau, H., and Grosveld, G. (1996). Interaction of cellular proteins with the leukemia-specific fusion proteins DEK-CAN and SET-CAN and their normal counterpart, the nucleoporin CAN. Oncogene *13*, 1801-1808.

Van Deursen, J., **Boer, J.**, Kasper, L., and Grosveld, G. (1996). G_2 arrest and impaired nucleocytoplasmic transport in mouse embryos lacking the proto-oncogene *CAN/Nup214*. EMBO J. *15*, 5574-5583.

Boer, J., Bonten-Surtel, J., and Grosveld, G. (1996). Growth arrest and apoptosis in myeloid cells overexpressing the oncoprotein CAN. Blood *88*, Suppl.1, 552a.

Caslini, C., Murti, K.G., Ashmun, R.A., Domer, P.H., Korsmeyer, S.J., **Boer, J.**, Grosveld, G., and Look, A.T. (1996). Subcellular localization and cell cycle effects of the MLL-AF4 fusion oncoprotein. Blood *88*, Suppl.1, 557a.

Boer, J., Mahmoud, H., Raimondi, S., Grosveld, G., and Krance, R. (1997). Loss of the *DEK-CAN* fusion transcript in a child with t(6;9) acute myeloid leukemia following chemotherapy and allogeneic bone marrow transplantation. Leukemia *11*, 299-300.

Boer, J., Van Deursen, J., Croes, H., Fransen, J., Grosveld, G. The nucleoporin CAN/Nup214 binds to both the cytoplasmic and the nucleoplasmic sides of the nuclear pore complex. Exp Cell Res, *in press*.

Boer, J., Bonten-Surtel, J., Grosveld, G. Overexpression of the nucleoporin CAN/NUP214 induces growth arrest, nucleocytoplasmic transport defects and apoptosis. Submitted.