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A rapid RT–PCR based method to isolate complementary DNA fragments flanking retrovirus integration sites

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ABSTRACT

Proto-oncogenes in retrovirally induced myeloid mouse leukemias are frequently activated following retroviral insertion. The identification of common virus integration sites (VISs) and isolation of the transforming oncogene is laborious and time consuming. We established a rapid and simple PCR based procedure which facilitates the identification of VISs and novel proto-oncogenes. Complementary DNA fragments adjacent to retrovirus integration sites were selectively isolated by applying a reverse transcriptase (RT) reaction using an oligo(dT)-adaptor primer, followed by PCR using the adaptor sequence and a retrovirus long terminal repeat (LTR) specific primer. Multiple chimeric cDNA fragments suitable for Southern and northern blot analysis were isolated.

Retroviral insertional mutagenesis is a powerful method to isolate proto-oncogenes from retrovirally induced leukemias and lymphomas (reviewed in ref. 1). A common VIS, which marks the position of a possible proto-oncogene, is characterized by retroviral insertions within corresponding genomic loci of various independent tumors and visualized by Southern blot analysis with probes flanking the actual VIS. Unknown flanking DNA sequences have been determined by genomic cloning (2), inverse PCR (3), biotinylated DNA labelling followed by PCR (4) and other PCR based methods (5,6). However, these methods carry certain disadvantages. Isolation of VIS flanking cellular DNA fragments by genomic cloning requires the establishment of genomic DNA libraries, which is time-consuming and the libraries have to be made for every individual tumor or cell line. The PCR based methods consist of critical ligation (3), tailing (5) or biotinylating steps (4).

Several mechanisms are known by which retroviral sequences affect normal gene expression (1). Promoter activation as well as enhancement by proviral integration within the 3′ untranslated region require that the viral LTR and the cellular gene are in the same transcriptional orientation (1). As a result of these integrations transcription may be initiated from the retroviral LTR promoters and terminated by polyA signals of cellular genes (Fig. 1A). Consequently, retrovirally initiated chimeric mRNA transcripts consist of a 5′ leader derived from the viral LTR (2,7) and a 3′ polyA tail of a cellular gene. The overall RT–PCR based method to isolate these chimeric cDNAs is schematically shown in Figure 1A. PolyA$^+$ RNA was purified using oligo(dT) cellulose columns (Pharmacia) from a panel of CasBrM-MuLV induced murine myeloid leukemia cell lines (NFS22, NFS56, NFS58, NFS60 and NFS78) (8). First strand cDNA was obtained by reverse transcriptase reactions at 37°C with 3 μg polyA$^+$ RNA, 1 U RNAguard (Pharmacia) and 100 U SuperScript RT (Gibco) in 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 0.5 mM dNTPs and 40 mM oligo(dT)-adaptor primer (5′-GTCGCGAATTCGTCGACGCG$(dT)_{15}$–3′). The integrity of the polyA$^+$ RNA and first strand cDNA synthesis was verified by PCR [10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 150 μM dNTPs and 2.5 U *Taq*-polymerase (Pharmacia), 1 min at 94°C, 1 min at 50°C and 3 min at 72°C (25 cycles)] with human β-actin specific primers, highly homologous to murine β-actin and located in two separate exons (MB6: 5′-CT-GGACTTCGAGCAAGAGAT-3′ and MB7: 5′-TCGTCATAC-TCCTGCTTGCT-3′). Fragments of 433 bp were amplified with the β-actin primers solely in the presence of reverse transcriptase (Fig. 1B). Subsequently, PCRs (1 min at 94°C, 1 min at 58°C and 3 min at 72°C for 30 cycles) were performed on the RT reactions of the NFS cell lines using the LTR specific primer (pLTR1: 5′-GGGTCTCCTCAGAGTGATTG-3′) and the adaptor primer (adaptor: 5′-GTCGCGAATTCGTCGACGCG-3′). Fragments of different size, 0.1–2.5 kb, were detected in all cell lines tested (Fig. 1B). No DNA fragments were amplified if PCR was not preceded by cDNA synthesis, indicating that only transcribed fragments were amplified. Fragments were cloned into the *Eco*RV site of pBluescript SK$^+$ (Statagene) and the viral origin of the cDNAs was confirmed by sequence analysis (Fig. 1C). No cDNAs were detected entirely consisting of viral sequences but, as expected, 100% of the cDNAs contained LTR sequences at the 5′ end. The cDNA sequences were compared with the database of the National Centre for Biotechnology Information (NCBI) to identify possible homologous sequences. VISs were detected within the murine homolog of the h*ERG* gene (9), which encodes a transcription factor, involved in AML t(16;21) (10) and within the promoter region of the mouse T-cell receptor γ chain near the *Vg6* gene (11).

The basis of insertional mutagenesis to identify proto-oncogenes is the isolation of common VISs in independent tumors or tumor cell lines. Southern blot analysis of one fragment, derived from NFS22, is shown in Figure 2A. Rearrangements in various independent cell lines, i.e., NFS22, NFS58, NFS60 and NFS78, were detected. A

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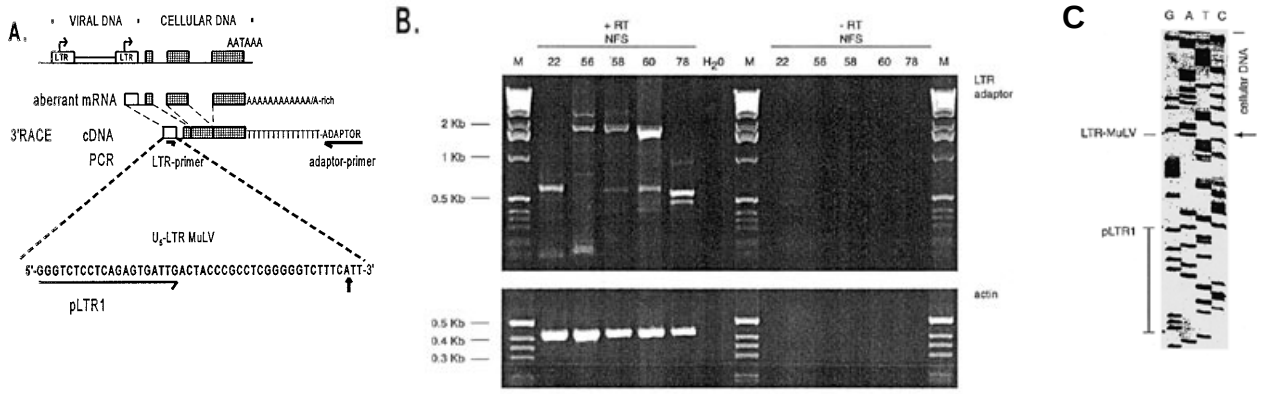

Figure 1. (**A**) Schematic representation of the strategy to rapidly amplify cDNA fragments adjacent to virus integration sites. Viral insertions in a cellular gene may generate aberrant mRNAs initiated through transcription from the viral promoter located on the long terminal repeat (LTR). The aberrant mRNA is polyadenylated downstream of the cellular polyadenylation signal (AATAAA). cDNA is synthesized by reverse transcription using an oligo(dT)-adaptor primer which primes on the polyA tails or A-rich sequences (3′ RACE, 3′ rapid amplification of cDNA ends). Using the adaptor primer and an LTR specific primer (pLTR1) the cellular DNA fragments flanking the VIS are amplified by PCR. (**B**) RT–PCR (adaptor/pLTR1) on polyA$^+$ RNA isolated from CasBrM-MuLV induced myeloid leukemia cell lines NFS22, NFS56, NFS58, NFS60 and NFS78 (+RT). As a control PCR was carried out on polyA$^+$ RNA samples without the addition of reverse transcriptase (–RT). The integrity of the isolated polyA$^+$ RNA and the first strand cDNA was confirmed by RT–PCR with β-actin primers (M, marker). (**C**) Sequence analysis of downstream of the LTR primer located MuLV LTR-specific sequences. The arrow (5′-TTTCA^NN-3′) indicates the general fusion site of viral and cellular cDNA in both A and C. The cDNA sequence depicted in this figure is identical to the murine EST (DDBJ/EMBL/GenBank accession no. W97251).

partial restriction map and the orientations of the proviruses were established (Fig. 2B) using the Southern blot data. The orientation and location of the proviral DNAs in NFS58, NFS60 and NFS78 implied that fragment 1 was localized in the commonly rearranged *Evi1* locus (7,12), within intron 2 of the *Evi1* gene. Northern blot analysis showed that this intron due to proviral integration is highly expressed in NFS22 and NFS78 (Fig. 2C), which could be expected from the orientation of virus integrations in NFS22 and NFS78 (Fig. 2B). In myeloid leukemias the *Evi1* gene is frequently activated due to viral integration within the murine gene (7) and in human AMLs due to translocation involving chromosome 3q26 (13).

Transcripts initiated from the retroviral promoter were selectively amplified. The existence of a polyA signal (AATAAA) (70%) and more than 15 adenine residues (55%) at the 3′ end of all cDNA fragments (data not shown) may suggest that cDNA synthesis regularly initiated at mRNA polyA tails. It is, however, possible that cDNA synthesis initiates at A-rich sequences. In fact, the isolated *Evi1* cDNA represents a sequence located 5′ within a cellular gene. Nevertheless, the isolation of cDNA fragments will eventually facilitate the identification of target genes in the leukemias (Fig. 2C). cDNA fragments which did not show homology with known genes will be used for Southern blot screening purposes to search for new common VISs. The fact that three out of 13 cDNAs represent known genes of which two (*Evi1* and *Erg*) have been demonstrated to be involved in myeloid leukemia (10,13) suggests that the described method is powerful for the rapid identification of novel common VISs and proto-oncogenes.

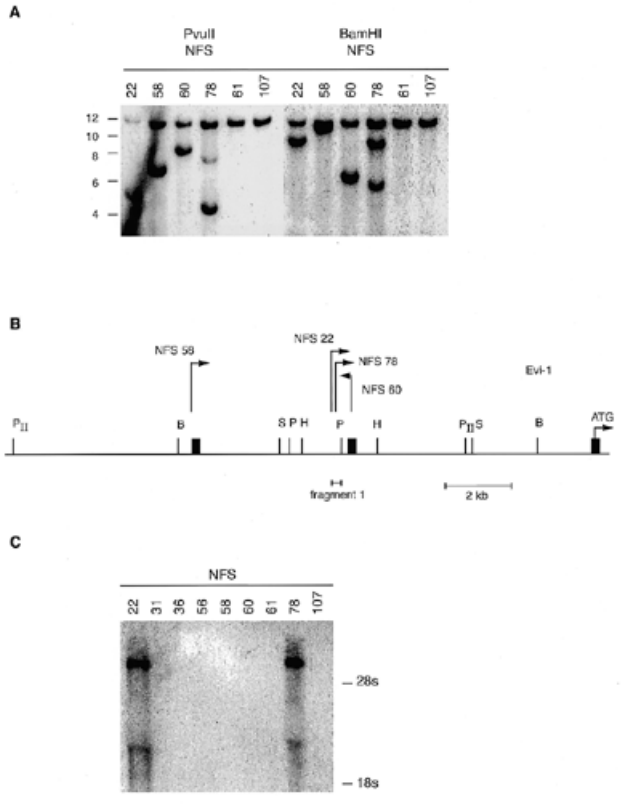

Figure 2. (**A**) Southern blot analysis (*Pvu*II and *Bam*HI digest) of a panel of retrovirally induced leukemic cell lines NFS22, NFS58, NFS60, NFS78, NFS61 and NFS107 hybridized with fragment 1. (**B**) Limited restriction map of the *Evi1* locus with the site and orientation of viral integrations, indicated by arrows, in the leukemic cell lines NFS22, NFS58, NFS60 and NFS78 (PII, *Pvu*II; B, *Bam*HI; S, *Sst*I; P, *Pst*I; H, *Hin*dIII). The black boxes indicate the 5′ end exon structure of the *Evi1* gene (7,12). (**C**) Northern analysis of the cell lines NFS22, NFS31, NFS36, NFS56, NFS58, NFS60, NFS61, NFS78 and NFS107 probed with fragment 1.

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