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Xeroderma pigmentosum group A correcting protein from calf thymus

A.P.M. Eker [a], W. Vermeulen [a], N. Miura [b], K. Tanaka [b], N.G.J. Jaspers [a], J.H.J. Hoeijmakers [a] and D. Bootsma [a]

[a] *MGC – Department of Cell Biology and Genetics, Erasmus University Rotterdam, Rotterdam, The Netherlands and* [b] *Institute for Molecular and Cellular Biology, Osaka University, Osaka 565, Japan*

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Summary

A proteinous factor was purified from calf thymus and HeLa cells, which specifically corrects the excision repair defect of xeroderma pigmentosum complementation group A (XP-A) cells. Recovery of UV-induced unscheduled DNA synthesis after microinjection of XP-A cells was used as a quantitative assay for the correcting activity of protein preparations. XP-A correcting protein appears to be very stable as it withstands heating to 100°C and treatment with SDS or 6 M urea. A molecular weight of 40–45 kD was found both under native (gel filtration) and denaturing (SDS-PAGE) conditions. Calf XP-A protein binds to single-stranded DNA more strongly than to double-stranded DNA, but shows no clear preference for UV-irradiated DNA. Polyclonal antibodies raised against human recombinant XP-A protein, which strongly inhibit UV-induced unscheduled DNA synthesis of normal human cells, completely abolished XP-A correcting activity when mixed with calf thymus preparations. This indicates a close relationship between human gene product and the calf protein. In the final preparation two main protein bands were present. Only one band at approx. 41 kD showed both DNA binding activity in Southwestern blots and immune reaction with human XP-A antibody, suggesting that this is the active calf XP-A correcting factor.

Xeroderma pigmentosum (XP) is a rare autosomal recessive human disease. The skin of XP patients is highly sensitive to UV light resulting in severe sunburn, abnormal pigmentation and a high incidence of skin cancer while in some cases neurological aberrations are found (for a general review see, e.g., Cleaver and Kraemer, 1989). In cells of most XP patients excision repair of damaged DNA is absent or much reduced (Cleaver, 1968), probably due to a defect in the first or incision step of this repair pathway (Cleaver, 1969; Setlow et al., 1969), resulting in a diminished DNA repair synthesis (unscheduled DNA synthesis, UDS).

Correspondence: A.P.M. Eker, Erasmus University Rotterdam, Department of Cell Biology and Genetics, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands.
Fax: 10-4360225.

Abbreviations: XP, xeroderma pigmentosum; UDS, unscheduled DNA synthesis; PBS, phosphate-buffered saline.

At present 7 excision-deficient XP complementation groups (XP-A to G) are known (Bootsma et al., 1989; Vermeulen et al., 1991). Patients falling into group A, one of the most common complementation groups, exhibit severe clinical symptoms with both skin and nervous system disorders. XP-A cells show a greatly diminished survival and low UDS (2–5% of normal cells) after UV irradation. No consensus exists on the exact biochemical nature of the repair defect in XP-A. Originally, the defect was thought to be located in the (UV-)endonuclease activity which makes the incision once a UV lesion has been recognized, see, e.g., a report on the absence of a specific endonuclease in XP-A cells (Kaufmann, 1988). Moreover, the repair defect can be efficiently reversed by micrococcal UV-endonuclease or phage T4 endonuclease V, introduced into XP cells either by microinjection (de Jonge et al., 1985; Yamaizumi et al., 1989) or by expression of the corresponding gene (Valerie et al., 1987; Colico et al., 1991). These endonucleases can, however, complement all XP groups and are thought to act as a general bypass enzyme not specific for XP-A. In contrast, the *E. coli* excision repair proteins UvrA, B, C, and D appear to be unable to complement the XP-A defect (Zwetsloot et al., 1986). After an early report that (UV-)endonuclease activity in XP-A cells is not impaired (Mortelmans et al., 1976; see also Kano and Fujiwara, 1983), additional evidence was recently obtained that these endonucleases are present in XP-A cells but probably cannot function unless a factor is added which is present in normal cells (Tsongalis et al., 1990).

Several attempts have been reported to isolate and identify an XP-A correcting factor using cytoplasmic microinjection (de Jonge et al., 1983; Yamaizumi et al., 1986; Vermeulen et al., 1986), electroporation (Tsongalis et al., 1990), transport into permeabilized cells (Keeney and Linn, 1990) or an in vitro repair assay (Wood et al., 1988), or by comparing enzymatic activities in normal and XP cells (Kuhnlein et al., 1983). Only total or slightly purified cell extracts were found to complement XP-A cells and as a consequence no definite conclusions concerning the nature of the XP-A correcting factor were reached.

Recently, a human and a homologous mouse gene have been cloned which after transfection correct the UV sensitivity of XP-A cells (Tanaka et al., 1989, 1990). Although this has led to the elucidation of the molecular defect in a number of XP-A patients (Satokata et al., 1990, 1992), the corresponding biologically active XP-A protein has not yet been isolated from mammalian sources. We report here, using microinjection as a quantitative repair assay, a more elaborate purification and characterization of XP-A correcting protein from calf thymus, permitting the recognition of a single protein band as the active correcting protein, and its relationship to the human XP-A gene. Part of these results have been summarized before (Hoeijmakers et al., 1990).

Materials and methods

Cell lines

Cell lines belonging to various XP complementation groups used in this study are listed in Table 3. Fibroblasts were cultured in Ham's F10 medium supplemented with 11% fetal calf serum and 100 IU penicillin and 100 μg streptomycin per ml.

Analysis of XP correcting activity

XP correcting activity was measured with a microinjection assay, for experimental details see de Jonge et al. (1983). In short, fibroblasts were fused with inactivated Sendai virus and cultivated for at least 3 days to allow extinction of S-phase cells, which could be confused with UDS-positive cells. Using a glass microneedle, fused cells were injected with cell extracts, followed by UV irradiation (15 J/m^2, 254 nm) and labeling with [^{3}H]-thymidine for 2 h. Unscheduled DNA synthesis (UDS) was measured autoradiographically after 3 days of exposure by counting silver grains above the nuclei. For routine determination of XP-A correcting activity fused XP25RO primary fibroblasts were used.

Purification of cell extracts

Extracts were prepared from cultured cells suspended in phosphate-buffered saline (PBS) by sonication followed by centrifugation to remove cell debris.

For large-scale purification calf thymus was collected as fresh as possible in the local slaughterhouse, frozen in pieces and stored at −80°C. The following purification steps were performed at 4°C. Thawed thymus was disintegrated in ice-cold PBS with an Ultraturrax disperser (Ika) for 5 periods of 30 s. The resulting suspension was centrifuged for 1 h at 130,000 × *g*. The supernatant was filtered through cheesecloth to remove fat and loaded on a porous silica column (spherosil 100–200 μm, 40–80 nm pore size, IBF) previously equilibrated in PBS. The column was washed with PBS and eluted with PBS containing 0.4 M NaCl and 6% polyethyleneglycol 6000. The eluate was diluted with an equal volume of buffer A (10 mM NaCl, 10 mM K-phosphate, pH 7.0) and loaded overnight on a blue-sepharose column. The column was washed with PBS containing 0.5 M NaCl and eluted with PBS containing 2 M NaCl. Saturated ammonium sulfate solution was added slowly to the eluate up to 50% saturation. After stirring for 30 min the precipitate was collected by centrifugation and dissolved in PBS containing 1 mM 2-mercaptoethanol. Preparations were frozen quickly in liquid nitrogen after addition of 20% (v/v) glycerol and stored at −80°C.

Essentially the same protocol was used for purification of XP-A correcting factor from HeLa cells.

XP-A protein from calf thymus was further purified by chromatofocusing (Polybuffer system, Pharmacia) on PBE 94 ion exchanger previously equilibrated with 25 mM histidine/HCl, pH 6.22. The column was eluted with PB74/HCl buffer diluted 1:8, pH 4.0. All solutions contained 6 M urea. Active XP-A protein, eluted at approx. pH 4.9, was concentrated by ultrafiltration (Millipore Ultrafree MC, 10 kD cut-off) prior to SDS gel electrophoresis (see below). Gel pieces were extracted with PBS yielding a highly purified XP-A protein preparation which was used for Southwestern analysis.

Protein concentrations were determined with the coomassie brilliant blue method (Bradford, 1975).

SDS-PAGE and DNA binding experiments

Partially purified XP-A protein was heated at 95°C for 5 min in the presence of 1% SDS and 280 mM 2-mercaptoethanol prior to SDS-polyacrylamide gel electrophoresis (PAGE) with prestained markers in neighboring lanes. After completion of electrophoresis gel pieces were cut out, crushed and extracted overnight with PBS containing 1 mM 2-mercaptoethanol. Supernatants were dialyzed (Pierce microdialyzer system 100) to remove excess SDS, prior to determination of biological activity with the microinjection assay.

For DNA binding (Southwestern analysis, Mazen et al., 1989) samples were subjected to SDS-gel electrophoresis and blotted wet to a nitrocellulose membrane (Schleicher & Schull BA83). Membranes were washed, blocked with non-fat milk and incubated with a ^{32}P-labeled 66-mer DNA fragment (Ménissier-de Murcia et al., 1989) in single- or double-stranded form for 1 h at 4°C. After washing autoradiography was performed. Then membranes were dyed with amido black, or incubated for immunodetection with polyclonal antibodies raised against, and highly reactive with, recombinant human XP-A protein (Miura et al., 1991) followed by incubation with alkaline phosphatase conjugated secondary antibody and appropriate dye development. This permitted the detection of DNA binding and protein bands or XP-A specific immune reaction on the same membrane.

DNA binding was also studied using chromatography on columns containing cellulose with covalently linked UV-irradiated calf thymus DNA or agarose containing entrapped single-stranded DNA (Eker and Fichtinger-Schepman, 1975).

DNA helicase and nuclease activity were estimated with an electrophoretic mobility shift assay using a partial duplex of M13 DNA and a ^{32}P-labeled 17-mer oligonucleotide (Tuteja et al., 1991).

In vitro translation

Human XP-A cDNA, cut at nucleotides −24 and 860 (Tanaka et al., 1990), was cloned behind a T7 promoter yielding plasmid pGEM.H19.WS containing the complete open reading frame, which was linearized by digestion with *Bam*HI endonuclease and in vitro transcribed and G-capped with T7 RNA polymerase for 45 min at 37°C in the presence of 0.5 mM ATP, CTP, UTP, GpppG and 0.05 mM GTP. Then additional RNA

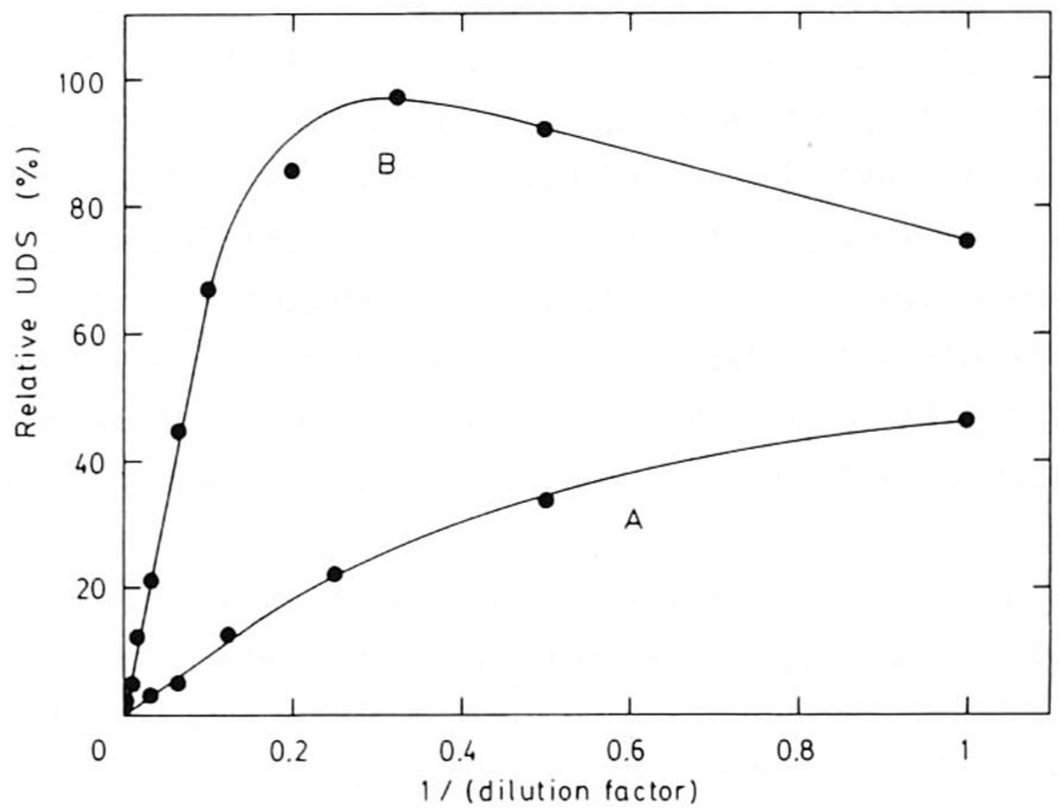

Fig. 1. Dilution curves of crude (curve A) and partially purified step 4 (curve B) calf thymus extracts. XP-A correcting activity was measured with the microinjection assay using XP25RO fibroblasts and is given as UDS (grains/nucleus) relative to normal C5RO cells (UDS = 100).

polymerase was added and incubation was continued for 30 min in the presence of 0.5 mM GTP. The resulting mRNA was in vitro translated into XP-A protein using nuclease-treated rabbit reticulocyte lysate (Promega).

Results

Quantitation of XP-A correcting activity with the microinjection assay

Microinjection into fused fibroblasts was used as a routine assay for determination of XP-A correcting activity in protein samples. Dilution curves for a crude and more purified preparation are shown in Fig. 1. With crude cell extracts a clear correction (curve A in Fig. 1), shown by the increase in UDS after UV irradiation of injected cells, was found although the wild-type UDS level was not reached. With a partially purified preparation the wild-type level was reached (curve B in Fig. 1), but injection of larger amounts resulted in a decrease in UDS, probably a consequence of inhibition of cellular (repair) processes due to the presence of contaminations. Most importantly, the initial parts of both dilution curves show a linear relationship between the amount of injected protein and UDS. This validates the use of the microinjection assay for quantitative determination of XP-A correcting activity, provided protein samples are suitably diluted to reach the linear part of the dilution curve.

Differences in the number of counted grains due to variations in the UDS or autoradiography protocol between separate experiments can be corrected by using the grain count of noninjected but identically treated fused XP-C cells as an internal standard. Normal cells are less useful due to the high grain count (> 200 grains/nucleus) obtained under our conditions.

Despite the fact that solutions to be assayed are injected directly into the cytoplasm of living cells, rather unphysiological conditions like 0.5 M NaCl, 1 M urea or 6% polyethyleneglycol 6000 do not fatally disturb the cells or inhibit repair synthesis. Ionic detergents like SDS proved to be harmful, however, even in low concentrations. Yet sufficient experimental freedom is left to use microinjection as a quantitative assay during purification of XP-A correcting activity.

Occurrence of XP-A correcting activity

For large-scale purification of XP-A correcting factor a suitable source material was needed. To examine whether mammalian tissues other than human could be used, we verified first whether bovine cells could complement the XP-A defect by interspecies cell fusion. In fused heterokaryons both types of nuclei showed a clear UV-induced UDS up to the level found in bovine cells (see Fig. 2), indicating efficient complementation.

Extracts of various cells and tissues were screened for the ability to correct the repair defect of XP-A cells. The highest activity was found in HeLa or K562 (human chronic myelogenous leukemia) cells, and bovine brain and thymus (Table 1). Other tissues showed a modest (human placenta) to marginal (e.g., liver) or no activity. The activity is not restricted to outer tissues (skin fibroblasts) but is even higher in several internal tissues which will never suffer from UV damage, suggesting that XP-A correcting activity is not confined to UV-induced lesions.

Because of the general availability, easy manipulation and high activity it was decided to use calf thymus as a source for isolation and purification of XP-A correcting activity.

TABLE 1

XP-A CORRECTING ACTIVITY IN DIFFERENT TISSUE AND CELL EXTRACTS

	Activity	Spec. Act.
Primary fibroblasts (human)	±	
Lung fibroblasts (calf)	±	
SV40 transformed fibroblasts (human)	+	
HeLa, K562 (human) cells	+ +	35–55
Placenta (human)	+	5.5
Liver (calf, rat, pig, monkey)	±	
Regenerating liver (rat)	±	
Thymus (calf)	+ +	10.5
Brain (bovine) cerebellum	+	1.4
cerebrum	+ +	11.2
Spleen (calf)	−	
Pancreas (calf)	−	
Kidney (dog)	−	

Activity was determined as induction of UDS in XP-A fibroblasts after microinjection of cell-free extracts and consecutive UV irradiation: − undiluted negative; ± undiluted sometimes positive, dependent on preparation; + undiluted positive; + + 10-fold diluted positive.
Specific activity is expressed as (grains/nucleus)/mg protein.

Purification of XP-A correcting protein

XP-A correcting activity was partially purified from calf thymus as indicated in Table 2. The step 4 preparation was very active in correcting the XP-A defect and retained its activity for years when kept frozen at −80°C. The same purification protocol up to step 4 was used, with comparable results, for HeLa cells (not shown).

Further purification was shown to be possible as XP-A correcting activity was found to bind, in addition to the chromatographic materials mentioned in Table 2, to phenyl-sepharose, phosphocellulose or ultragel P, heparin-sepharose and

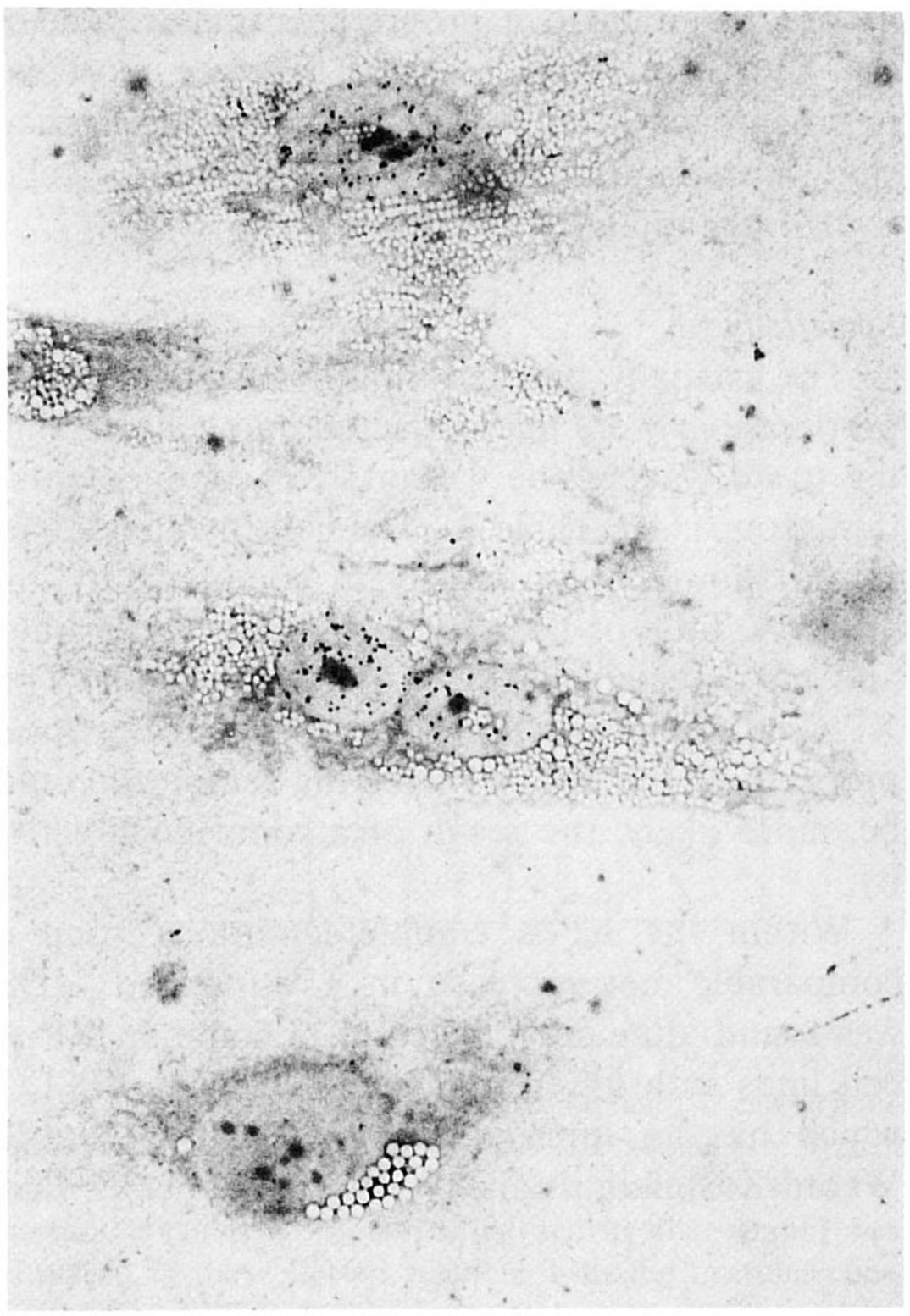

Fig. 2. Complementation of XP-A defect by fusion with calf cells. XP-A (XP25RO) fibroblasts (lower part, labeled with large spheres) show almost no UDS after UV irradiation while in 1DV calf skin primary fibroblasts (upper part, labeled with small spheres) high UDS is found. In fused cells (middle, containing both small and large spheres) both types of nuclei show UDS up to the level found in calf cells.

Q-sepharose. However, in most attempts hardly any increase of specific activity was obtained although substantial amounts of protein were re-

TABLE 2

PURIFICATION OF XP-A CORRECTING PROTEIN FROM CALF THYMUS

Purification step	Volume (ml)	protein (mg)	Total Act.	Spec. Act.	Purification (times)	Yield (%)
1. Crude extract	266	5265	55365	10.5	(1)	(100)
2. Porous silica	224	1489	58341	39.2	4	105
3. Blue-sepharose	51	78	41691	535	51	75
4. $(NH_4)_2SO_4$ dial.	10	60	39565	659	63	71
5. Red-sepharose	22	8.3	11000	1319	126	20
6. Hydroxyl-apatite	8	0.8	6824	8747	833	12

Total activity is expressed as: (grains/nucleus)×(dilution factor)×(volume). Specific activity is: (total activity)/(mg protein).

moved, pointing to a progressive inactivation of the XP-A correcting factor. Therefore most experiments were done with the partially purified step 4 preparation which was available in relatively large quantities.

Specificity

The partially purified calf thymus and HeLa preparations were microinjected into cells belonging to all (except the variant) XP complementation groups (see Table 3). The calf thymus preparation showed high activity in complementation group A, little or no activity in group C cell lines and no detectable activity in other groups. The XP-C complementation might be an intrinsic property of the XP-A correcting factor or could be, more likely, the result of a copurifying activity.

Within the XP-A complementation group a comparable net increase in UV-induced UDS was found after microinjection in common XP-A cell lines with low residual UDS and in XP8LO which has an unusual high residual UDS (de Weerd-Kastelein et al., 1976). This indicates that the biochemical defect is probably the same in these XP-A cell lines, irrespective of their different residual UDS levels.

In contrast with the calf thymus preparations, HeLa extracts purified up to and including step 4 were able to increase UDS in all complementation groups except B. This indicates that the HeLa preparation, unlike that from calf thymus, probably contains several proteins specific for various complementation groups. The lack of complementation by calf thymus extract in groups B, D, E, F and G also indicates that the high activity found in group A is not due to some aspecific activity comparable to micrococcal or T4 UV-endonuclease, which can correct all complementation groups (de Jonge et al., 1985) by bypassing the defective (pre)incision step.

Stability

Calf thymus XP-A correcting factor (step 4) appeared to be very stable. Solutions heated for 5 min at 100°C still retained part of their activity after cooling, despite an apparent loss of activity by coprecipitation with the bulk of proteins. The

TABLE 3

UV-INDUCED UDS IN XP FIBROBLASTS AFTER MICROINJECTION OF PARTIALLY PURIFIED (STEP 4) CALF THYMUS OR HeLa EXTRACTS

Complementation group	Cell line	Reference	Increase in UDS after microinjection of	
			Calf thymus extract	HeLa extract
A	XP25RO (low UDS)	Kraemer et al., 1975	+ + +	+ + +
	XP2CA (low UDS)	Hashem et al., 1980	+ + +	n.d.
	XP8LO (high UDS)	de Weerd-Kastelein, 1975	+ + +	n.d.
B	XP11BE	Kraemer et al., 1975a	−	−
C	XP2KA	W. Keijzer, unpublished	+	n.d.
	XP21RO	Kleijer et al., 1973	+	n.d.
	XP1TE	de Weerd-Kastelein, 1977	+	+
	XP20MA (former I)	Fischer et al., 1985 Bootsma et al., 1989	+	n.d.
D	XP1BR	W. Keijzer, unpublished	−	+ / + +
	XPCS2 (former H)	Moshell et al., 1983 Vermeulen et al., 1991	−	n.d.
E	XP2RO	de Weerd-Kastelein, 1974	−	+ +
F	XP126LO	Norris et al., 1988	−	+ +
G	XP2BI	Keijzer et al., 1979	−	+ +
−	C5RO (wild-type)		−	−

− undiluted negative; + undiluted positive; + + 10-fold diluted positive; + + + 100-fold diluted positive; n.d., not determined.

activity is also resistant to high salt (2 M NaCl), 6 M urea and detergents, both ionic (SDS) and nonionic (polyethyleneglycol, Triton X-100, Tween). It is, however, not clear whether the XP-A factor remains in an active form or rapidly renatures either in vitro or in the cell after microinjection when harsh conditions are removed.

The high stability raised some doubts about the proteinous nature of this factor. Therefore the partially purified preparation was incubated with proteinase K immobilized with CNBr-activated sepharose-4B (de Jonge et al., 1983). A gradual decrease in correcting activity was found which was absent when BSA-loaded sepharose beads were used instead, leaving no doubt about the proteinous character of the XP-A correcting factor.

Molecular weight

The molecular weight of the XP-A correcting protein was determined under denaturing and native conditions. Gel filtration using a sephacryl-200HR column yielded a value of approx. 45 kD (Fig. 3). At higher sample loads some activity was found at higher molecular weight, indicating a tendency to aggregate. Chromatography in the presence of 0.15% Triton X-100 to diminish molecular interactions gave the same molecular weight (not shown), pointing to a monomer configuration in dilute solutions.

The resistance towards heating and SDS permitted the use of SDS-gel electrophoresis for determination of molecular weight under denaturing conditions in partially purified samples. Extracts obtained from gel pieces cut out according to molecular weight intervals were used for estimation of XP-A correcting activity and re-electrophoresis (Fig. 4). Main activity was found in the 40–45 kD region, which indicates, together with the results of gel filtration experiments, that XP-A protein consists of a single protein chain. Some activity was found in the 30–40 kD region, indicating that likely partial degradation had taken place without complete loss of biological activity. Due to the large number of protein bands in this region no definitive assignment of a band to XP-A correcting activity could be made at this stage.

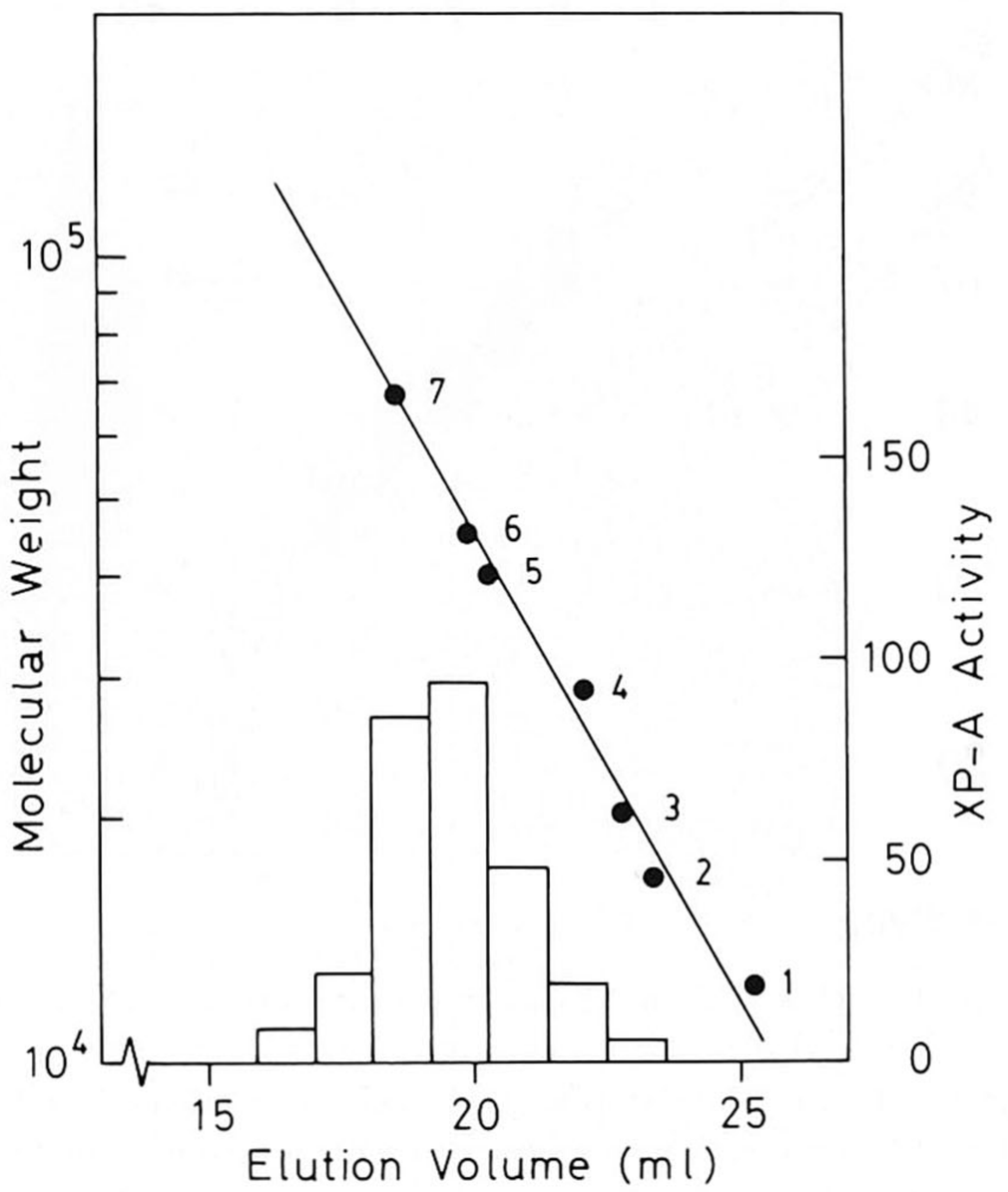

Fig. 3. Molecular weight determination of XP-A correcting protein by gel filtration. Partially purified (step 4) calf thymus extract was loaded on a sephacryl-200 HR column (1 × 50 cm), which was eluted with PBS containing 1 mM 2-mercaptoethanol. XP-A correcting activity (bar graph) was measured in the collected fractions with the microinjection assay using XP25RO fibroblasts. Molecular weight markers are cytochrome *c* (1), myoglobin (2), soy bean trypsin inhibitor (3), carbonic anhydrase (4), β-lactoglobulin (5), ovalbumin (6) and bovine serum albumin (7).

Essentially the same molecular weight was found for XP-A correcting activity from HeLa cells (not shown).

DNA binding

The biochemical defect in the excision repair pathway in XP cells is probably located either in the incision or in the pre-incision step. Therefore it is interesting to investigate DNA binding properties of XP-A protein. Partially purified XP-A protein was applied to single-stranded DNA agarose and UV-DNA cellulose columns (see Fig. 5). Activity was bound to both columns at low ionic strength, and could be eluted at approximately the same salt concentration (0.25–0.3 M NaCl), indicating that, although XP-A protein does bind to DNA, specific binding to UV lesions is apparently absent.

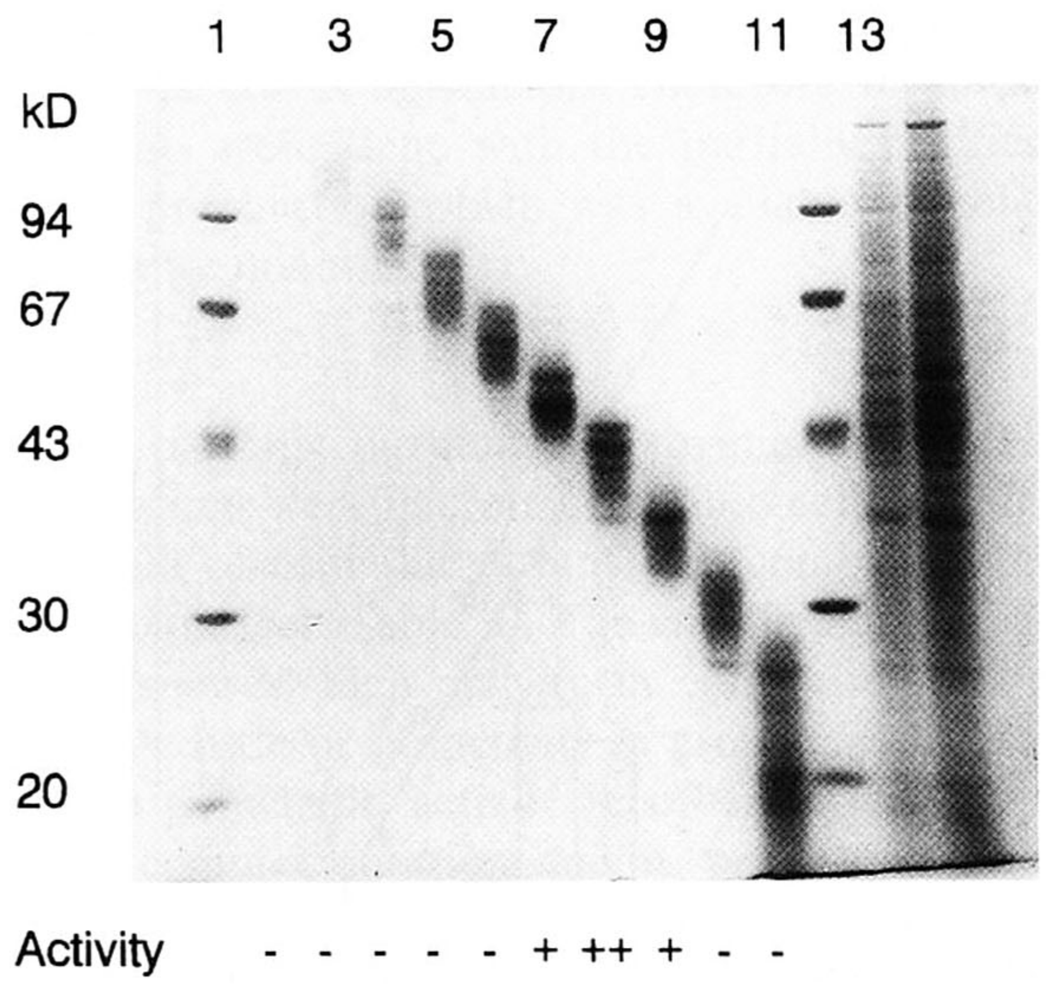

Fig. 4. SDS-PAGE of XP-A correcting protein. Partially purified (step 4) calf thymus extract (lanes 13 and 14) was subjected to SDS-PAGE. Pieces of gel were crushed and extracted; the resulting supernatants were microinjected to determine biological activity and in part again subjected to SDS-PAGE to obtain the molecular weight intervals. In lanes 2–11 the relationship is shown between molecular weight and XP-A correcting activity. Lanes 1 and 12 contain molecular weight markers.

DNA binding was also studied by Southwestern blot analysis. Highly purified XP-A protein (step 4, followed by chromatofocusing and SDS-PAGE, see Materials and methods) was subjected to SDS gel electrophoresis and blotted to a nitrocellulose membrane. Pieces of membrane were incubated with ^{32}P-labeled single-stranded, double-stranded or UV-irradiated double-stranded oligonucleotide probes followed by autoradiography. In all cases a single labeled band of approx. 41 kD was found (Fig. 6). Comparison of the intensity of these bands indicates that XP-A protein binds more strongly to single-stranded than to double-stranded DNA, while there is no increase in binding when UV lesions are present. After dyeing the membrane, only two protein bands with molecular weight 41–43 kD were visible, one of which corresponded with the DNA binding band.

DNA helicase and/or nuclease activity was detected in several purification steps mentioned in Table 2. On prolonged purification, however, no correlation was found between XP-A correcting and helicase or nuclease activity (not shown).

Relation to human XP-A gene product

Analysis of the product of the in vitro transcribed human XP-A gene revealed the presence of two protein bands with molecular weight 41–42 kD (Fig. 6, rightmost part), i.e., comparable with the bands found in the most purified calf XP-A protein preparation. This suggests a close rela-

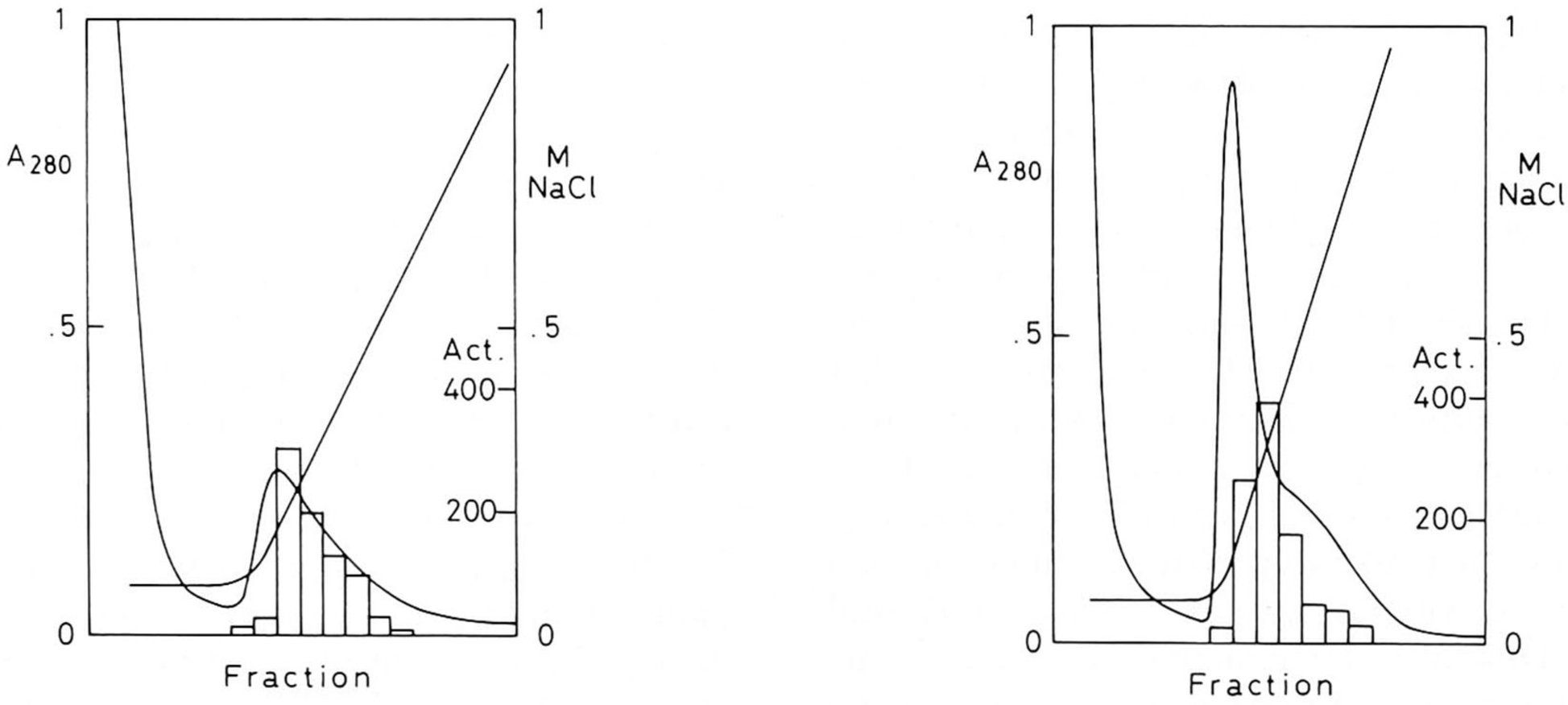

Fig. 5. DNA binding of XP-A correcting protein. Partially purified (step 4) calf thymus extracts were loaded to single-stranded DNA agarose (left panel) and UV-irradiated DNA cellulose (right panel). Columns were eluted with a NaCl gradient and XP-A correcting activity (bar graph) was determined with the microinjection assay using XP25RO fibroblasts.

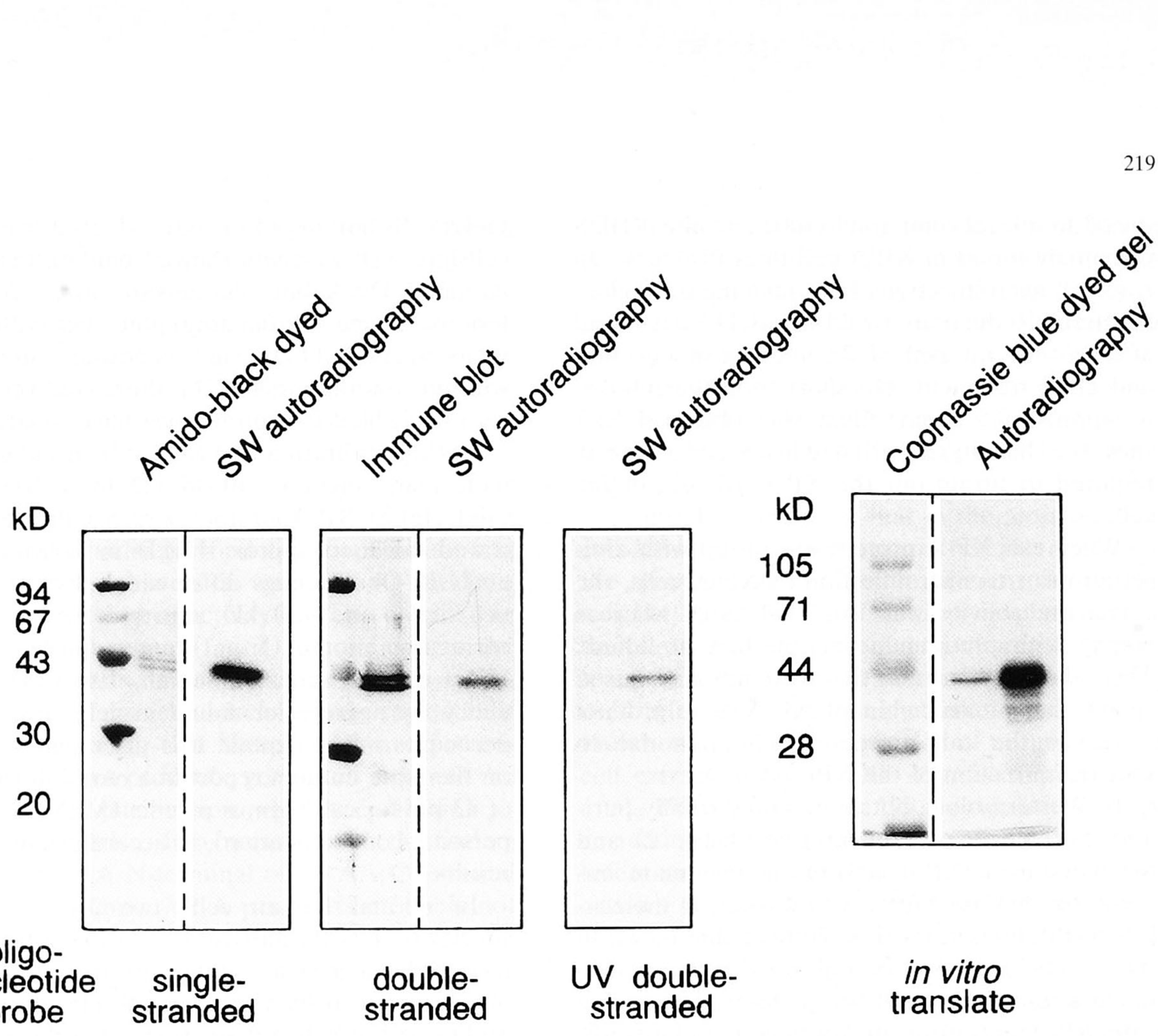

Fig. 6. Southwestern blot analysis of XP-A correcting protein. Highly purified XP-A correcting protein was subjected to SDS-PAGE and blotted on a nitrocellulose membrane which was subsequently incubated with different ^{32}P-labeled oligonucleotide probes (identical specific labeling). After autoradiography the membranes were either dyed or incubated with human XP-A protein-specific antiserum for immune analysis. In the rightmost part SDS gel electrophoresis of in vitro translated human XP-A gene is shown (in the middle lane no additional GTP was present during the second incubation period of the in vitro transcription reaction, see Materials and methods).

tionship between calf protein and the human gene product. In order to establish this relationship, antibodies raised against a recombinant protein derived from the human XP-A gene were used. When this antiserum was microinjected into normal human cells, UV-induced UDS was re-

TABLE 4

INFLUENCE OF HUMAN XP-A ANTIBODIES ON UDS

	UDS (grains/nucleus)
UDS measured 22 h after microinjection of antiserum into normal (C5RO) fibroblasts	
not injected	71
pre-immune serum (10-fold diluted)	70
XP-A antiserum (10-fold diluted)	5
UDS measured after microinjection of calf thymus (step 4) XP-A protein/antibody mixtures into XP-A (XP25RO) fibroblasts	
XP-A protein	66
XP-A protein + pre-immune (10-fold diluted)	64
XP-A protein + antibody (300-fold diluted)	2

Figures are corrected for residual UDS.

duced to a level comparable to the residual UDS commonly found in XP-A cell lines (Table 4). In contrast, microinjection of pre-immune serum had no effect. Reduction of wild-type UDS was found after a time interval of 22 h between injection and UDS treatment. On shortening the interval to approx. 0.5 h no effect was observed (not shown). This suggests that considerable time is required to titrate out the XP-A protein in the cell.

When calf XP-A protein was mixed with antiserum prior to microinjection in XP-A cells, the correcting activity was lost (Table 4), whereas mixing with pre-immune serum had no effect. These data demonstrate that the antibodies raised against human recombinant XP-A gene product recognize the calf protein which gives rise to specific correction of the XP-A defect.

In Western blots, obtained with partially purified calf XP-A protein (step 4, Table 2) and incubated with XP-A antiserum, two main immune bands were found with 40–42 kD molecular weight, together with several weaker bands in the 30–40 kD region (not shown). With pre-immune serum these bands were absent. Incubation with XP-A antiserum of Southwestern blots obtained with the most purified calf preparation resulted in two strong immune bands, comparable to the protein bands found on the dyed membrane, one of which corresponded to the 41 kD DNA binding band (Fig. 6). The results of both microinjection and immune blot experiments establish the close relationship between calf protein and human gene product and strongly indicate that the 41 kD protein band shown in Fig. 6 is the biologically active bovine equivalent of human XP-A protein.

Discussion

We have isolated and partially characterized a proteinous factor from calf thymus which is highly active in correction of the repair defect of XP-A cells. The properties of this protein apparently do not agree with proposed XP-A correcting factors or activities (Keeney and Linn, 1990; Yamaizumi et al., 1986; Kaufmann and Briley, 1988), except for a DNA binding activity described by Kuhnlein et al. (1983) present in human fibroblasts and HeLa cells but absent or reduced in several XP-A cell lines. This activity showed binding to single-stranded DNA but had no specificity for UV lesions. Since chromatographic behavior and molecular weight are in reasonable agreement with our findings, this SSB protein could be identical with the XP-A protein we have isolated.

Both gel filtration and SDS-PAGE indicated a molecular weight of 40–45 kD for calf thymus (and HeLa) XP-A protein, which could be narrowed down to approx. 41 kD in Southwestern analysis. Our findings differ widely from the values of 90 and 160 kD reported for an XP-A correcting protein from human placenta and HeLa cells (Yamaizumi et al., 1986). The tendency for aggregation found in gel filtration experiments might explain this discrepancy. Later on the same authors reported a molecular weight of 43 kD for calf thymus protein (M. Yamaizumi, personal communication), in accordance with our results.

In normal human cells two XP-A mRNA species of 1.3–1.4 and 1.0–1.1 kb have been detected (Tanaka et al., 1990), probably the result of alternative polyadenylation. While the size of 41 kD of the calf XP-A protein hardly fits into these mRNA sizes, there is a still larger discrepancy with the value of 31 kD deduced from the open reading frame of the human XP-A gene. At present no convincing and experimentally verified explanation is available for this discrepancy. The deduced amino acid sequence of the XP-A gene suggests the presence of a zinc finger, although it remains to be proven that XP-A protein contains Zn^{2+} and the zinc finger is indeed involved in DNA binding. It is likely that the mere presence of a zinc finger is not sufficient to explain the molecular weight aberration. In the C-terminal part of the XP-A gene two additional cysteine residues are present which might be involved, through Zn^{2+} coordination, in the binding of another XP-A protein molecule as has been found for, e.g., the HIV tat (Frankel et al., 1988) and T4 gene 32 (Nadler et al., 1990) proteins. It is even conceivable that the calf thymus XP-A protein we have isolated has in fact a dimer structure which is resistant against denaturation.

When we accept that the putative zinc finger is involved in DNA binding of XP-A protein, the

biological activity still present after denaturing treatment seems to indicate that this finger is either resistant against denaturation or rapidly and efficiently renatures afterwards. The presence of a zinc finger alone can hardly explain the high stability of XP-A protein: other structural properties, such as the high α-helical content suggested by the deduced secondary structure, might play a major role.

Although the main part of XP-A correcting activity in partially purified calf preparations was found to have a molecular weight of 40–45 kD, a significant amount of activity was also detected in the 30–40 kD region (Fig. 4). In addition to the 41–43 kD bands human XP-A antibodies recognized several discrete bands in this region (not shown) suggesting that these bands are proteolytic breakdown products, generated either in the cell or during purification, which are still biologically active. This is in agreement with the finding that the 58 AA N-terminal or 47 AA C-terminal part of the human XP-A protein is probably not essential for biological activity. This follows from the observation that an incomplete c-DNA lacking an N-terminal stretch is still capable of correcting the UV sensitivity of XP-A cells (Tanaka et al., 1990), and the discovery of a nonsense mutation which could give rise to a C-terminal truncated but likely biologically active protein in patient XP39OS who does not have the severe skin and neurological symptoms characteristic of the XP-A complementation group (Satokata et al., 1992).

Antibodies raised against human XP-A protein showed a clear affinity to calf XP-A protein in immune blots (Fig. 6) and inactivated this protein (Table 4), supporting the close relationship between calf and human XP-A protein. On injection of antibodies into normal cells a striking reduction of UV-induced UDS was found. Apparently normal human fibroblasts could, at least temporarily, be turned into XP-like cells by XP-A protein-specific antibodies. Remarkably, no immediate effect on UDS was found, but 22 h after microinjection the reduction of UDS was almost complete, indicating that factors other than a fast antigen-antibody recognition play a role. Several explanations are possible. Assuming that XP-A protein is located in the nucleus (Miura et al., 1991), it might take a long time for antibodies to penetrate into the nucleus and inactivate the protein. Or, when antibody remains in the cytoplasm where it will inactivate de novo synthesized XP-A protein, one has to wait for the decay of XP-A protein already present in the nucleus to see an effect on UDS. Alternatively, antibody in the cytoplasm might slowly deplete the nucleus of XP-A protein.

A puzzling finding is that in the most purified calf XP-A protein preparation two protein bands are present with slightly different molecular weights (Fig. 6). The simplest explanation is doubtless the presence of a contaminating protein which copurifies in the purification scheme used. However, comparable protein bands were found on immune precipitation of normal human cell extracts using the XP-A antiserum (Tanaka et al., 1990; Miura et al., 1991) as well as in XP-A correcting protein preparations purified by a different protocol (R.D. Wood, personal communication). Moreover, in vitro translation of the cloned human XP-A gene also yielded two discrete protein bands in the same molecular weight region (Fig. 6), suggesting that the proteins in these bands are encoded by the same gene and therefore are probably closely related. Since one of the bands does not show DNA binding (Fig. 6), this might represent the primary translated protein, which, in a posttranslational process (e.g., formation of zinc finger or proteolytic modification), is converted into the active, DNA binding form. No definite clues are available at this moment, however, to discriminate between various possibilities.

Both column chromatography and Southwestern blot experiments indicate that calf XP-A protein has DNA binding properties. This is in agreement with the presence of the putative zinc finger (see above) as well as possible helix-turn-helix motifs in the deduced secondary structure of the human XP-A gene (Tanaka et al., 1990). Under the conditions used no specific binding to UV lesions could be detected. However, Southwestern blots (Fig. 6) revealed a preference for single- over double-stranded DNA. This was not found in chromatographic experiments (Fig. 5) but, due to the way of preparation, the UV-DNA cellulose used will contain single-stranded re-

gions. This makes it inappropriate for discrimination between single- and double-strand specific binding. In chromatographic experiments no correlation was found between helicase or nuclease and XP-A correcting activity, in agreement with the apparent absence of putative nucleotide binding or helicase domains in the deduced amino acid sequence of the XP-A gene. Our results indicate therefore that XP-A protein is probably a single-stranded DNA binding protein without clear preferential binding to UV lesions.

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References

Bootsma, D., W. Keijzer, E.G. Jung and E. Bohnert (1989) Xeroderma pigmentosum complementation group XP-I withdrawn, Mutation Res., 218, 149–151.

Bradford, M.M. (1975) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem., 72, 248–254.

Cleaver, J.E. (1968) Defective repair replication of DNA in xeroderma pigmentosum, Nature (London), 218, 652–656.

Cleaver, J.E. (1969) Xeroderma pigmentosum: a human disease in which an initial stage of DNA repair is defective, Proc. Natl. Acad. Sci. (U.S.A.), 63, 428–435.

Cleaver, J., and K.H. Kraemer (1989) Xeroderma pigmentosum, in: C.R. Scriver et al. (Eds.), The Metabolic Basis of Inherited Disease, 6th edn., Vol. II, McGraw-Hill, New York, pp. 2949–2971.

Colicos, M.A., Y. Haj-Ahmed, K. Valerie, E.E. Henderson and A.J. Rainbow (1991) Construction of a recombinant adenovirus containing the *den*V gene from bacteriophage T4 which can partially restore the DNA repair defect in xeroderma pigmentosum fibroblasts, Carcinogenesis, 12, 249–255.

de Jonge, A.J.R., W. Vermeulen, B. Klein and J.H.J. Hoeijmakers (1983) Microinjection of human cell extracts corrects xeroderma pigmentosum defect, EMBO J., 2, 638–641.

de Jonge, A.J.R., W. Vermeulen, W. Keijzer, J.H.J. Hoeijmakers and D. Bootsma (1985) Microinjection of *Micrococcus luteus* UV-endonuclease restores UV-induced unscheduled DNA synthesis in cells of 9 xeroderma pigmentosum complementation groups, Mutation Res., 150, 99–105.

de Weerd-Kastelein, E.A., W. Keijzer and D. Bootsma (1974) A third complementation group in xeroderma pigmentosum, Mutation Res., 22, 87–91.

de Weerd-Kastelein, E.A., W. Keijzer, M. Sabour, J.M. Parrington and D. Bootsma (1976) A xeroderma pigmentosum patient having a high residual activity of unscheduled DNA synthesis after UV is assigned to complementation group A, Mutation Res., 37, 307–312.

de Weerd-Kastelein, E.A., W. Keijzer, G. Rainaldi and D. Bootsma (1977) Induction of sister chromatid exchanges in xeroderma pigmentosum cells after exposure to ultraviolet light, Mutation Res., 45, 253–261.

Eker, A.P.M., and A.M.J. Fichtinger-Schepman (1975) Studies on a DNA photoreactivating enzyme from *Streptomyces griseus* II. Purification of the enzyme, Biochim. Biophys. Acta, 378, 54–63.

Fischer, E., W. Keijzer, H.W. Thielmann, O. Popanda, E. Bohnert, L. Edler, E.G. Jung and D. Bootsma (1985) A ninth complementation group in xeroderma pigmentosum, XP I, Mutation Res., 145, 217–225.

Frankel, A.D., D.S. Bredt and C.O. Pabo (1988) Tat protein from human immunodeficiency virus forms a metal-linked dimer, Science, 240, 70–73.

Hashem, N., D. Bootsma, W. Keijzer, A. Greene, L. Coriell, G. Thomas and J.E. Cleaver (1980) Clinical characteristics, DNA repair and complementation groups in xeroderma pigmentosum patients from Egypt, Cancer Res., 40, 13–18.

Hoeijmakers, J.H.J., A.P.M. Eker, R.D. Wood and P. Robins (1990) Use of in vivo and in vitro assays for the characterization of mammalian excision repair and isolation of repair proteins, Mutation Res., 236, 223–238.

Kano, Y., and Y. Fujiwara (1983) Defective thymine dimer excision from xeroderma pigmentosum chromatin and its characteristic catalysis by cell-free extracts, Carcinogenesis, 4, 1419–1424.

Kaufmann, W. (1988) In vitro complementation of xeroderma pigmentosum, Mutagenesis, 3, 373–380.

Kaufmann, W.K., and L.P. Briley (1988) UV-endodeoxyribonuclease activities in diploid human fibroblasts, in: E.C. Friedberg and P.C. Hanawalt (Eds.), Mechanisms and Consequences of DNA Damage Processing, Liss, New York, pp. 257–261.

Keeney, S., and S. Linn (1990) A critical review of permeabilized cell systems for studying mammalian DNA repair, Mutation Res., 236, 239–252.

Keijzer, W., N.G.J. Jaspers, P.J. Abrahams, A.M.R. Taylor, C.F. Arlett, B. Zelle, H. Takebe, P.D.S. Kinmont and D. Bootsma (1979) A seventh complementation group in excision-deficient xeroderma pigmentosum, Mutation Res., 62, 183–190.

Kleijer, W.J., E.A. de Weerd-Kastelein, M.L. Sluyter, W. Keijzer and D. Bootsma (1973) UV-induced DNA repair

synthesis in cells of patients with different forms of xeroderma pigmentosum and heterozygotes, Mutation Res., 20, 417–438.

Kraemer, K.H., E.A. de Weerd-Kastelein, J.H. Robbins, W. Keijzer, S.F. Barrett, R.A. Petinga and D. Bootsma (1975) Five complementation groups in xeroderma pigmentosum, Mutation Res., 33, 327–340.

Kraemer, K.H., H.G. Coon, R.A. Petinga, S.F. Barrett, A.E. Rahe and J.H. Robbins (1975a) Genetic heterogeneity in xeroderma pigmentosum groups and their relationship to DNA repair rates, Proc. Natl. Acad. Sci. (U.S.A.), 72, 59–63.

Kuhnlein, U., S.S. Tsang, O. Lokken, S. Tong and D. Twa (1983) Cell lines from xeroderma pigmentosum complementation group A lack a single-stranded-DNA-binding activity, Biosci. Rep., 3, 667–674.

Mazen, A., J. Ménissier-de Murcia, M. Molinete, F. Simonin, G. Gradwohl, G. Poirier and G. de Murcia (1989) Poly(ADP-ribose)polymerase: a novel finger protein, Nucleic Acids Res., 17, 4689–4698.

Ménissier-de Murcia, J., M. Molinete, G. Gradwohl, F. Simonin and G. de Murcia (1989) Zinc-binding domain of poly(ADP-ribose)polymerase participates in the recognition of single strand breaks on DNA, J. Mol. Biol., 210, 229–233.

Miura, M.N., I. Miyamoto, H. Asahina, I. Satokata, K. Tanaka and Y. Okada (1991) Identification and characterization of xpac protein, the gene product of the human XPAC (xeroderma pigmentosum group A complementing) gene, J. Biol. Chem., 266, 19786–19789.

Mortelmans, K., E.C. Friedberg, H. Slor, G. Thomas and J.E. Cleaver (1976) Defective thymine dimer excision by cell-free extracts of xeroderma pigmentosum cells, Proc. Natl. Acad. Sci. (U.S.A.), 73, 2757–2761.

Moshell, A.N., M.B. Ganges, M.A. Lutzner, H.G. Coon, S.F. Barrett, J.M. Dupuy and J.H. Robbins (1983) A new patient with both xeroderma pigmentosum and Cockayne syndrome establishes the new complementation group H, in: Cellular Responses to DNA Damage, UCLA Symposia on Molecular and Cellular Biology, New Series, Vol. 11, Liss, New York, pp. 209–213.

Nadler, S.G., W.J. Roberts, Y. Shamoo and K.R. Williams (1990) A novel function for zinc(II) in a nucleic acid-binding protein. Contribution of zinc(II) toward the cooperativity of bacteriophage T4 gene 32 protein, J. Biol. Chem., 265, 10389–10394.

Norris, P.G., J.L.M. Hawk, J.A. Avery and F. Giannelli (1988) Xeroderma pigmentosum complementation group F in a non-Japanese patient, J. Am. Acad. Dermatol., 18, 1185–1188.

Satokata, I., K. Tanaka, N. Miura, I. Miyawata, Y. Satoh, S. Kondo and Y. Okada (1990) Characterization of a splicing mutation in group A xeroderma pigmentosum, Proc. Natl. Acad. Sci. (U.S.A.), 87, 9908–9912.

Satokata, I., K. Tanaka, N. Miura, M. Narita, T. Mimaki, Y. Satoh, S. Kondo and Y. Okada (1992) Three nonsense mutations responsible for group A xeroderma pigmentosum, Mutation Res., 273, 193–202.

Setlow, R.B., J.D. Regan, J. German and W.L. Carrier (1969) Evidence that xeroderma pigmentosum cells do not perform the first step in the repair of ultraviolet damage to their DNA, Proc. Natl. Acad. Sci. (U.S.A.), 64, 1035–1041.

Tanaka, K., I. Satokata, Z. Ogita, T. Uchida and Y. Okada (1989) Molecular cloning of a mouse DNA repair gene that complements the defect of group-A xeroderma pigmentosum, Proc. Natl. Acad. Sci. (U.S.A.), 86, 5512–5516.

Tanaka, K., N. Miura, I. Satokata, I. Miyamoto, M.C. Yoshida, Y. Satoh, S. Kondo, A. Yasui, H. Okayama and Y. Okada (1990) Analysis of a human DNA excision repair gene involved in group A xeroderma pigmentosum containing a zinc finger domain, Nature (London), 348, 73–76.

Tsongalis, G.J., W.C. Lambert and M.W. Lambert (1990) Electroporation of normal human DNA endonucleases into xeroderma pigmentosum cells corrects their DNA repair defect, Carcinogenesis, 11, 499–503.

Tuteja, N., K. Rahman, R. Tuteja and A. Falaschi (1991) DNA helicase IV from HeLa cells, Nucleic Acids Res., 19, 3613–3618.

Valerie, K., A.P. Green, J.K. de Riel and E.E. Henderson (1987) Transient and stable complementation of ultraviolet repair in xeroderma pigmentosum cells by the *den* V gene of bacteriophage T4, Cancer Res., 47, 2967–2971.

Vermeulen, W., P. Osseweyer, A.J.R. de Jonge and J.H.J. Hoeijmakers (1986) Transient correction of excision repair defects in fibroblasts of 9 xeroderma pigmentosum complementation groups by microinjection of crude human cell extracts, Mutation Res., 165, 199–206.

Vermeulen, W., M. Stefanini, S. Giliani, J.H.J. Hoeijmakers and D. Bootsma (1991) Xeroderma pigmentosum complementation group H falls into complementation group D, Mutation Res., 255, 201–208.

Wood, R.D., P. Robins and T. Lindahl (1988) Complementation of the xeroderma pigmentosum DNA repair defect in cell-free extracts, Cell, 53, 97–106.

Yamaizumi, M., T. Sugano, H. Asahina, Y. Okada and T. Uchida (1986) Microinjection of partially purified protein factor restores DNA damage specifically in group A of xeroderma pigmentosum cells, Proc. Natl. Acad. Sci. (U.S.A.), 83, 1476–1479.

Yamaizumi, M., T. Inaoka, T. Uchida and E. Ohtsuka (1989) Microinjection of T4 endonuclease V produced by a synthetic *den*V gene stimulates unscheduled DNA synthesis in both xeroderma pigmentosum and normal cells, Mutation Res., 217, 135–140.

Zwetsloot, J.C.M., A.P. Barbeiro, W. Vermeulen, H.M. Arthur, J.H.J. Hoeijmakers and C. Backendorf (1986) Microinjection of *Escherichia coli* UvrA, B, C, and D proteins into fibroblasts of xeroderma pigmentosum complementation groups A and C does not result in restoration of UV-induced unscheduled DNA synthesis, Mutation Res., 166, 89–98.

Note

During the process of reviewing the manuscript two papers were published by other groups con-

cerning purification and characterization of XP-A correcting protein from calf thymus (Robins et al., 1991; Sugano et al., 1991). Both publications largely confirm our findings with respect to molecular weight, high stability of the protein and occurrence of a doublet band in SDS-PAGE. However, while Sugano et al. (1991) mention the absence of specific binding of XP-A protein to UV-damaged DNA, like we found, Robins et al. (1991) report a 1000-fold preference in binding to UV-irradiated over nondamaged DNA. Although the assay conditions used (e.g., nature and concentration of salts) are different, we have no satisfactory explanation at the moment and additional binding experiments are necessary to resolve this discrepancy.

Robins, P., C.J. Jones, M. Biggerstaff, T. Lindahl and R.D. Wood (1991) Complementation of DNA repair in xeroderma pigmentosum group A cell extracts by a protein with affinity for damaged DNA, EMBO J., 10, 3913–3921.

Sugano, T., T. Uchida and M. Yamaizumi (1991) Identification of a specific protein factor defective in group A xeroderma pigmentosum cells, J. Biochem. (Tokyo), 110, 667–674.