Biochemistry

 Volume 25, Number 1 January 14, 1986

Articles

Cross-Linking of Cytokeratins to DNA in Vivo by Chromium Salt and *cis*-Diamminedichloroplatinum(II)†

Andrzej Wedrychowski, Warren N. Schmidt, W. Steven Ward, and Lubomir S. Hnilica*

Departments of Biochemistry and Pathology, Center in Molecular Toxicology, and A. B. Hancock, Jr., Memorial Laboratory, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

Received May 21, 1985

ABSTRACT: The in vivo cross-linking of cytokeratins to DNA in intact Novikoff ascites hepatoma cells exposed to the chromium salt K_2CrO_4 and *cis*-diamminedichloroplatinum(II) (*cis*-DDP) was studied. Cytokeratin–DNA complexes were obtained by high-speed centrifugation of cells solubilized in buffered 4% sodium dodecyl sulfate. The cytokeratins were identified electrophoretically and immunologically by use of polyclonal and monoclonal antibodies. Time dependence experiments showed that detectable cross-linking occurred after cells were exposed to K_2CrO_4 for at least 4 h, and the amount of keratin–DNA complexes increased with the incubation time. Each of the three Novikoff ascites hepatoma cytokeratins (p39, p49, and p56) showed a different apparent rate of cross-link formation with DNA. Cytokeratin–DNA complexes were detectable in our system only with K_2CrO_4 concentrations of 200 μM or greater, and saturation in cross-linking was effected at approximately 2 mM. Higher K_2CrO_4 concentrations (up to 5 mM) did not produce further significant increases in the amount of cross-linked cytokeratins. Chromium and *cis*-DDP cross-linked the same cytokeratins at approximately the same ratios; however, both agents cross-linked the major cytokeratins selectively, since not all cytokeratins present in Novikoff hepatoma cell lysates could be cross-linked to DNA. Further evidence of DNA–cytokeratin complexes was obtained by CsCl gradient centrifugation. Our results document the ability of chromate and *cis*-DDP to produce DNA–cytokeratin cross-links in vivo and show that in live Novikoff hepatoma cells some, but not all, of the components of intermediate filaments are within cross-linking distance of DNA.

Intermediate filaments (8–10 nm), an integral part of almost every mammalian cell type, are composed of well-defined protein species of restricted tissue distribution. In epidermal and almost all other epithelial cells the intermediate filaments are made of cytokeratins that form a heterogeneous family of proteins of approximately 40–70 kDa, which are poorly soluble in most physiological solvents (Lazarides, 1982; Weber & Geisler, 1984). Using poly- and monoclonal antibodies to cytokeratins, several investigators were able to show that in epithelial cells these proteins can be divided into families of mutually exclusive species that associate to form the intermediate filament network and change with differentiation and carcinogenesis (Battifora et al., 1980; Bannasch et al., 1980; Schlegel et al., 1980; Franke et al., 1981b; Fuchs et al., 1984; Sun et al., 1984; Geiger et al., 1984; Rheinwald et al., 1984).

We have reported in Novikoff hepatoma and other transplantable carcinomas the presence of three proteins, p39, p49, and p56 (approximately 39, 49, and 56 kDa), which coisolated with chromatin and were subsequently identified as cytokeratins (Fujitani et al., 1978; Schmidt et al., 1981, 1982). While p56 was found in normal rat hepatocytes, the other two cytokeratins appeared limited to most rodent carcinomas (Schmidt & Hnilica, 1982) and, according to our most recent observations, to the simple or ductular epithelia of bile ducts, colonic mucosa, mammary glands, etc. (Schmidt et al., 1984).

Because, as other keratins, p39, p49, and p56 are poorly soluble in physiological solvents, their presence in isolated chromatin may represent a preparation artifact. To investigate the possibility that intermediate filaments form a continuous

† This research was supported by NCI Grants CA 26412 and CA 36479.

* Correspondence should be addressed to this author at the Department of Biochemistry, Vanderbilt University School of Medicine.

0006-2960/86/0425-0001$01.50/0

structure entering the cell nucleus, we have exposed Novikoff hepatoma cells to two DNA–protein cross-linking agents, *cis*-DDP[1] and K_2CrO_4, and analyzed the proteins cross-linked to DNA with the aid of several poly- and monoclonal antibodies specific for the hepatoma cytokeratins. We show here that some, but not all, Novikoff hepatoma cytokeratins are within cross-linking distance of both the cross-linking agents employed in our experiments.

Materials and Methods

Materials. *cis*-DDP was purchased from Sigma Chemical Co. (St. Louis, MO). K_2CrO_4 was from Fisher Scientific Co. (Pittsburgh, PA). DNase I and RNase A were from Worthington Biochemical Corp. (Freehold, NJ). Electrophoresis supplies were from Bio-Rad Laboratories (Richmond, CA).

Novikoff ascites hepatoma (NAH) cells were passed in male Sprague-Dawley rats (Harlan Industries, Indianapolis, IN) every 5 days. Cells were harvested, resuspended in ice-cold 0.17 M NH_4Cl to lyse contaminating erythrocytes, washed in PBS, and used freshly in the cross-linking experiments.

Cross-Linking Experiments. Identical procedures were employed for cross-linking with *cis*-DDP and K_2CrO_4. The washed NAH cells were resuspended in 20 volumes (packed cell/buffer, v/v) of Hank's balanced salt solution containing different concentrations of cross-linking agent and incubated at 37 °C for indicated time intervals. During incubation the cells were gently shaken every 20 min. The Hank's balanced solution contains, in addition to isotonic concentrations of NaCl and *d*-glucose, significant amounts of Ca^{2+}, Mg^{2+}, and K^+ ions. Because of their dissociated states, these cations can react with chromium anions in the incubation medium. Their reaction products are soluble, however, and should enter the hepatoma cells with ease. The 1:20 ratio of cells to incubation medium was found optimal. Other ratios, e.g., 1:50, were also used with identical results. At each collection time, the viability of NAH cells was determined by the Trypan Blue exclusion assay. It should be mentioned that the dye exclusion assay does not provide a measure of long-term cytotoxicity. It is being used, in our experiments, to gain information about the integrity of cellular membranes in the presence of K_2CrO_4 or *cis*-DDP. Indeed, with the exception of the lowest metal concentrations, all the employed conditions would result in cell death during long-term incubation assays (Bradley et al., 1982; Köpf-Maier et al., 1983; Cupo & Wetterhahn, 1984; Bakke et al., 1984). We are using, in this study, K_2CrO_4 and *cis*-DDP as cross-linking reagents to detect associations of various cytokeratins with DNA, neglecting long-term cytotoxicity of the compounds.

Next, the cells were washed twice in ice-cold PBS and solubilized in 4% SDS–50 mM Tris-HCl, pH 7.5 (4% SDS buffer). From this step on, all the solutions also contained 1 mM phenylmethanesulfonyl fluoride (PMSF). The solution was stirred slowly at room temperature for 4 h and then gently homogenized in a glass homogenizer with a loosely fitting Teflon pestle (Ward et al., 1984). To remove insoluble cellular debris the homogenized solution was centrifuged at 770*g* for 10 min. The pellets were discarded, and the supernatant was centrifuged at 100000*g* for 16 h. After centrifugation at 100000*g* for 16 h, the resulting DNA pellets were rinsed with 4% SDS buffer and resuspended in 5 M urea (volume equal to the origianl 4% SDS solution). After being stirred at 4 °C for 3 h, the resulting solution was gently homogenized, SDS was added to the final concentration of 4% (urea–SDS buffer), and the solution was stirred slowly at room temperature for 1 h. After centrifugation at 100000*g* for 16 h, the final pellets were rinsed with urea–SDS buffer (or urea–4% sarcosyl buffer for CsCl gradient centrifugation) and then resuspended in 2 mM Tris-HCl buffer, pH 7.5. The suspension was sonicated in 2 mM Tris-HCl buffer, pH 7.5 (three 20-s bursts followed by intermittent cooling on ice), precipitated with ice-cold acetone, and resuspended in 2 mM Tris-HCl–1 mM $MgCl_2$ buffer, pH 7.5. Next, DNase I was added (25 μg/mL, sp act. 1872 units/mg), and the samples were incubated at 37 °C for 1 h in preparation for SDS–polyacrylamide gel electrophoresis.

Polyacrylamide Gel Electrophoresis and Immunoassays. The nuclease-digested samples were made (final concentration) 2% SDS, 10% glycerol–5% 2-mercaptoethanol–0.0625 M Tris-HCl, pH 6.8, boiled for 5 min, and electrophoresed as described (Laemmli, 1970) by using a 3.0% stacking gel and a 7.5% running gel. The separated proteins were visualized either by using the silver staining technique (Wray et al., 1981) or by transferring the proteins to nitrocellulose sheets as described by Towbin et al. (1979). To visualize the cytokeratin antigens, the nitrocellulose sheets were incubated with appropriate antiserum and stained by the peroxidase–antiperoxidase (PAP) procedure of Sternberger (1979). With mouse monoclonal antibodies, an affinity-purified rabbit antimouse immunoglobin was used together with the rabbit PAP staining technique (Schmidt et al., 1984). The immunoreactive staining was quantitated densitometrically following the method of Guengerich et al. (1982).

Antisera. To identify the cytokeratins cosedimenting with the DNA after incubation of NAH cells with K_2CrO_4 or *cis*-DDP, antisera to keratins were elicited in rabbits. Monoclonal antibodies were obtained exactly as described by Schmidt et al. (1984). Polyclonal antiserum X207 recognized all three major cytokeratins p39, p49, and p56. Monoclonal antibody E3 recognized specifically cytokeratin p39 (Schmidt et al., 1984), and monoclonal antibody B9 recognized cytokeratin p56 and several lower molecular weight cytokeratins, except the p39. Monoclonal antibody C7 reacted with p56 and several lower molecular weight cytokeratins with a specificity similar to but not identical with that of the B9 monoclonal antibody.

Preparation of Cytokeratin-Enriched Fractions. Freshly collected NAH cells were incubated for 10 min on ice in 0.17 NH_4Cl to lyse contaminant red blood cells and washed twice with PBS (800*g* for 10 min). Packed NAH cells (20 mL) were resuspended in 180 mL of 10 mM Tris-HCl, pH 7.4–1 mM PMSF (from this step on, all solutions contained 1 mM PMSF), briefly homogenized, and sonicated for three 20-s bursts. DNase I (1872 units/mg) and RNase A (75 units/mg) were each added to 50 μg/mL, $MgCl_2$ was added to 2 mM, and the solution was incubated on ice for 1 h. Next, 180 mL of 4 M NaCl–0.2% Nonidet NP-40–10 mM Tris-HCl, pH 7.4, was added to the homogenate. The resulting solution was homogenized and centrifuged at 140000*g* for 1 h. The pellets were resuspended in 2 M NaCl–0.1% Nonidet NP-40–10 mM Tris-HCl, pH 7.4, and centrifuged again at 140000*g* for 1 h. The resulting pellets were solubilized in 30 mL of 8 M urea–25 mM HEPES–25 mM 2-mercaptoethanol, pH 7.4, stirred for

[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; NAH, Novikoff ascites hepatoma; PBS, phosphate-buffered saline, 10 mM Na_2HPO_4, 150 mM NaCl; PAP, peroxidase–antiperoxidase; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; 4% SDS buffer, 4% SDS–50 mM Tris-HCl, pH 7.5; urea–SDS buffer, 4% SDS–50 mM Tris-HCl, pH 7.5–5 M urea; *cis*-DDP, *cis*-diamminedichloroplatinum(II); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; kDa, kilodaltons.

3 h at room temperature, and centrifuged at 200000*g* for 1 h. The pellets were discarded, and the supernatant was dialyzed against 2 mM Tris-HCl, pH 7.4–1 mM PMSF (with three changes) at 4 °C for 24 h. The sample was lyophilized and prepared for SDS–polyacrylamide gel electrophoresis.

CsCl Equilibrium Density Gradient Centrifugation. The NAH cells were incubated with K_2CrO_4, solubilized in SDS buffer, and centrifuged as described above. The resulting DNA-containing pellets were resuspended in 5 M urea–4% sarcosyl solution, stirred slowly at room temperature for 4 h, and then gently homogenized in a glass homogenizer with a loosely fitting Teflon pestle. The resulting suspension was centrifuged at 100000*g* for 16 h. The final pellets were washed with 2 mM Tris-HCl–1 mM PMSF, pH 7.5, and resuspended by gentle homogenization in CsCl solution (1.7 g/mL); the suspension was divided among six centrifuge tubes and centrifuged at 100000*g* for 78 h. Half-milliliter fractions were collected from each gradient, and their density and absorbances at 260 nm were determined. Corresponding fractions from each gradient were combined, dialyzed against 2 mM Tris-HCl–0.1 mM PMSF, pH 7.5, lyophilized, and electrophoresed.

Whole Cell Lysates. The cells were resuspended in 0.17 M NH_4Cl for 10 min to lyse contaminating red blood cells. Next, the cells were washed twice in PBS and resuspended in 10 mM Tris-HCl, pH 7.4, with 1 mM PMSF. After 10 min the solution was sonicated by two 20-s bursts with an intermediate cooling period on ice. Next, 25 μg/mL each of DNase I and RNase A was added (2540 and 75 units/mg, respectively), the solution was incubated on ice for 1 h, and the sample was then prepared for SDS gel electrophoresis.

Results

Novikoff ascites hepatoma cells tolerated incubation with K_2CrO_4 and *cis*-DDP well. The Trypan Blue exclusion assay indicated 84% average survival for chromium-treated cells and 83% survival for *cis*-DDP-treated cells. The average viability for control cells was 86%. Even at the highest concentrations of metals used (5 mM for K_2CrO_4, 1 mM for *cis*-DDP) and after 8 h of incubation, the average viabilities did not fall below 83% and 82%, respectively. We realize that the K_2CrO_4 concentrations used for the cross-linking experiments were high and certainly toxic to the cells. These concentrations, however, did not damage the cell membrane (as shown by a Trypan Blue exclusion assay) nor did they cause any other visible damage to the cells. The need for the higher K_2CrO_4 concentration may be explained by the fact that chromium can first be coordinated to small organic ligands (Sanderson, 1976a,b). Because of the relatively small amounts of protein that became associated with the DNA pellets in cells treated with both metals (especially chromium), we employed a sensitive silver stain technique (Wray et al., 1981) for the detection of proteins resolved by polyacrylamide gel electrophoresis. Since we have previously reported studies with *cis*-DDP-induced DNA–protein cross-links (Banjar et al., 1984; Ward et al., 1984), we used this metal for comparison to the chromium experiments.

Figure 1A, lanes 1–5, shows a time-dependent rise in DNA–protein cross-links during incubation (up to 8 h) in 1 mM K_2CrO_4. It is apparent that the cytokeratins p39, p49, and p56 clearly distinguishable in lane 6 (cytoskeletal protein enriched preparation; Schmidt et al., 1984) were not among the major proteins cross-linked by chromium (lanes 3–5).

Figure 1B represents an immunoblot of the gel from Figure 1A stained with a polyclonal rabbit antiserum raised to all NAH cytokeratins. The first detectable cross-linking occurred after 4 h of incubation of cells with K_2CrO_4, and it is evident

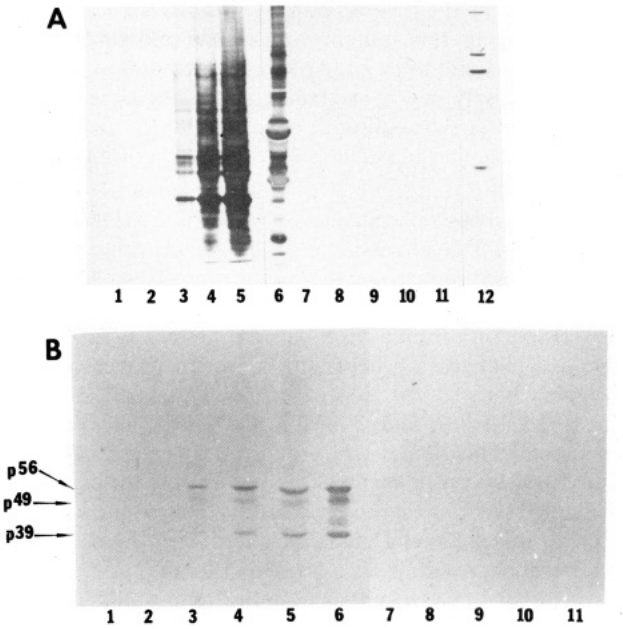

FIGURE 1: Time dependence of DNA–protein cross-linking in 1 mM K_2CrO_4. (A) Equal amounts of NAH cells were incubated for 1, 2, 4, 6, and 8 h with 1 mM K_2CrO_4. Cells were then solubilized directly in SDS-containing buffer, and the DNA was separated by high-speed ultracentrifugation as described under Materials and Methods. Following electrophoresis, protein bands were stained with $AgNO_3$. Lanes 1–5 represent NAH cells incubated with K_2CrO_4 for 1, 2, 4, 6, and 8 h, respectively (10 μg/lane as DNA). Lane 6 represents the cytoskeletal protein enriched fraction (5 μg of protein/lane). Lanes 7–11 represent controls of NAH cells incubated without K_2CrO_4 for 1, 2, 4, 6, and 8 h, respectively (10 μg/lane as DNA). Lane 12 represents molecular weight standards (Bio-Rad) (myosin, M_r 200000; β-galactosidase, M_r 116000; phosphorylase B, M_r 94000; ovalbumin, M_r 43000). (B) Time-dependent cross-linking of cytokeratins p39, p49, and p56 to DNA in the presence of 1 mM K_2CrO_4. Immunoblot of lanes 1–11 (gel in panel A) was incubated with rabbit polyclonal antiserum raised to total cytokeratins in NAH cells (Schmidt & Hnilica, 1982). Lanes 1–11 are the same as in panel A.

Table I: Time-Dependent DNA Cross-Linking of NAH Cytokeratins p39, p49, and p56

time of incubation with 1 mM K_2CrO_4 (h)	cytokeratin cross-linked (%)[a]		
	p39	p49	p56
1–2	nd[b]	nd	nd
4	8	34	38
6	62	82	89
8	100	100	100

[a] Immunoblot from Figure 1B was densitometrically scanned, and the percent of each cross-linked cytokeratin was calculated for the individual time points assuming the cross-linking at 8 h to be 100%. [b] nd, not detectable.

that the three cytokeratins were being cross-linked differentially. There was no significant increase in cross-linking after 8 h of incubation, and the cells became less viable. Consequently, the 8-h incubation time was adopted in all our experiments. Lane 6 in Figure 1B shows the cytoskeletal protein enriched preparation containing the three major cytokeratins and other proteins (possibly other unidentified cytokeratins or proteolytic degradation products), which are not detectable in lanes 3–5. The faint, irregular band seen in most lanes in Figure 1B at approximately 64 kDa represents contamination of the electrophoretic system with human keratin (Ochs, 1983).

Lanes 1–5 from Figure 1B were scanned densitometrically, and for each cytokeratin the OD after the eighth hour of incubation (Figure 1B, lane 5) was expressed as 100% (Table I). Only 8% of cytokeratin p39 was cross-linked after the

fourth hour of incubation; however, at the sixth hour the cross-linking of this protein increased 6-fold to 62%. In contrast, cytokeratins p49 and p56 were cross-linked 34% and 38%, respectively, at 4 h, and their cross-linking increased only about 2-fold at the sixth hour of incubation. This suggests different apparent rates of cross-link formation for these three cytokeratins.

Figure 2 shows dependence of the amount of DNA–cytokeratin cross-link formation on the K_2CrO_4 concentration. Following electrophoresis, the resolved proteins were transferred to nitrocellulose and incubated with the same anti-NAH cytokeratin antiserum as in Figure 1B. Lanes 1–8 in part A represent high-speed pellet fractions from cells incubated respectively with 10 μM, 50 μM, 100 μM, 200 μM, 300 μM, 400 μM, 500 μM, and 1 mM K_2CrO_4 for 8 h. The first detectable indication of DNA–cytokeratin cross-linking appeared at a concentration of 200 μM K_2CrO_4 in the incubation medium (lane 4 in Figure 2A). From then on the intensity of cross-linked protein bands increased with the chromium concentration, resulting, at 1 mM K_2CrO_4, in the appearance of background reactivity evident in the immunoblot shown in Figure 2C (each lane represents a protein concentration equivalent to 10 μg of DNA in the pelleted material). Interestingly, the three cytokeratins displayed different K_2CrO_4 concentration dependences for protein–DNA cross-linking. p49 and p56 were detectable in DNA-containing pellets at 200 μM K_2CrO_4 (lane 4), and their relative amounts increased almost linearly with increasing chromium concentration. In contrast, cytokeratin p39 was detected only at a chromium concentration of 400 μM (lane 5), and even then its intensity was much lower than those of p49 and p56.

Figure 2B represents evaluation of the densitometric scans of lanes 1–8 from Figure 2A. It is evident that p49 and p56 were cross-linked to DNA in a fairly linear fashion over the course of the experiment. Cytokeratin p39, however, required a higher initial K_2CrO_4 concentration to form cross-links with DNA than p49 and p56 (Figure 2B). Further increase of K_2CrO_4 concentration (1–5 mM, Figure 2C) did not result in more DNA–cytokeratin complexes.

Figure 2D shows the evaluation of densitometric scans of lanes 1–6 from Figure 2C. All the three cytokeratins reached saturation of complex formation at K_2CrO_4 concentrations between 2 and 3 mM. At these higher K_2CrO_4 concentrations, polyclonal antiserum recognized other high molecular weight proteins, possibly protein–protein cross-links, resulting in higher background. The three major cytokeratins were still well stained and identifiable, however. The apparent differences in the staining intensities (Figure 2A, lane 7, vs. Figure 2C, lane 1, and Figure 2A, lane 8, vs. Figure 2C, lane 2) were due to the different optimal camera settings for the negatives as well as different settings for the prints. The actual intensity of the bands in the immunoblots was identical.

To confirm the differential concentration dependence and specificity of DNA–cytokeratin cross-link formation, duplicate immunoblots as in Figure 2A were stained with monoclonal antibody to p56 cytokeratin, which also reacts with several lower molecular weight cytokeratins but not with p39 (Ward et al., 1985), and with monoclonal antibody specific for cytokeratin p39 (Schmidt et al., 1984). Although the antibody to p56 reacted with other putative cytokeratin species (Figure 3A, lane 9), the DNA pellets of cross-linked cells exhibited only the p56 antigen (lanes 1–8). Apparently, these cytokeratins, although present in the enriched cytoskeletal preparation, did not cross-link to the DNA in detectable amounts. The distribution of reactive antigens in Figures 2 and 3 also

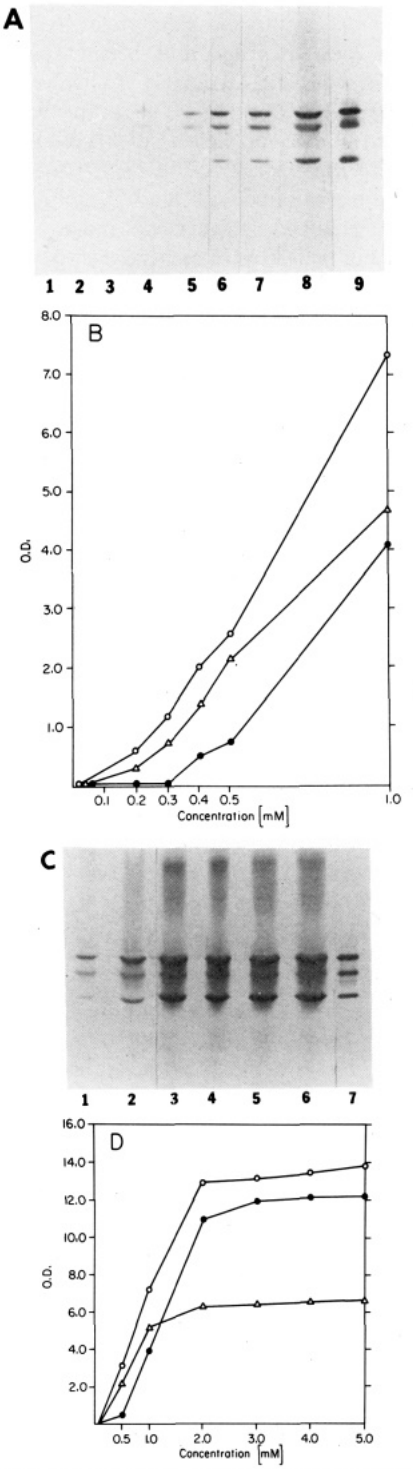

FIGURE 2: Dependence of DNA–cytokeratin cross-linking on K_2CrO_4 concentration. (A) Equal numbers of NAH cells were incubated with 10 μM, 50 μM, 100 μM, 200 μM, 300 μM, 400 μM, 500 μM, and 1 mM K_2CrO_4 for 8 h (lanes 1–8, respectively, loaded 20 μg/lane as DNA). Lane 9 represents the cytoskeletal protein enriched fraction (5 μg/lane). After cross-linking, cells were solubilized in SDS buffer, and the DNA was separated by ultracentrifugation as described under Materials and Methods. The resolved proteins (gel not shown) were transferred to nitrocellulose and reacted with the same anti-NAH cytokeratin antiserum as in Figure 1B. (B) Differential amounts of cytokeratin–DNA cross-link formation. Lanes 1–8 from panel A were scanned densitometrically and expressed in OD units. O, p56; Δ, p49; ●, p39. (C) Dependence of DNA–cytokeratin cross-linking on K_2CrO_4 concentration. Equal numbers of NAH cells were incubated with 0.5, 1, 2, 3, 4, and 5 mM K_2CrO_4 for 8 h (lanes 1–6) and solubilized as described under panel A. Lane 7 represents the cytoskeletal protein enriched fraction. (D) Lanes 1–6 from panel C were scanned densitometrically as in panel B and expressed in OD units. O, p56; Δ, p49; ●, p39.

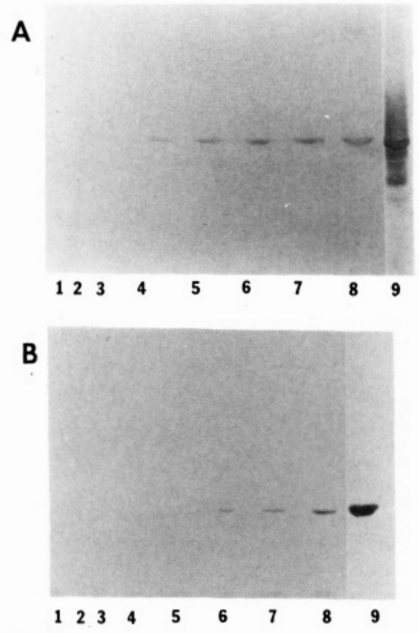

FIGURE 3: Duplicate immunoblots as shown in Figure 2A were incubated with monoclonal antibody B9 to cytokeratin p56 or with monoclonal antibody E3 to cytokeratin p39 (panels A and B, respectively, 15 μg/lane as DNA). Lane 9 in panels A and B represents the cytoskeletal protein enriched preparation at 0.5 μg/lane.

shows little detectable protein–protein cross-linking or rearrangements of the cytokeratins resulting from the exposure of cells to K_2CrO_4 at concentrations of 1.0 mM or lower.

Finally, we compared the cross-linking ability and selectivity of *cis*-DDP and K_2CrO_4 using several antibodies of different specificities (Figure 4). Concentrations of 1 mM and incubation times of 8 h were used for both metals, since these values were assumed by us to be optimal. From these experiments *cis*-DDP appears to be a stronger cross-linker than K_2CrO_4, and the comparison of lanes 1 and 4 of Figure 4A indicates that *cis*-DDP cross-linked more proteins, especially of higher molecular weights. Lane 5 of this figure shows no detectable cross-linked protein in cells incubated without metal.

Figure 4 (panels B–E) presents immunoblots of duplicate gels as shown in panel A reacted with (B) polyclonal antiserum to the NAH cytokeratin preparation, (C) monoclonal antibody to p39, (D) monoclonal antibody C7 to p56, and (E) monoclonal antibody B9 to p56 cytokeratins. As can be seen, the polyclonal antiserum reacted with all the three principal NAH cytokeratins, i.e., the p39, p49, and p56 antigens. It also reacted with an additional protein band slightly above the p49 antigen. Only traces of this protein can be seen in the cytoskeletal protein preparation and none in the *cis*-DDP or K_2CrO_4 cross-linked DNA pellets (lanes B1 and B4). The antibody to p39 is monospecific while both the C7 and B9 antibodies reacted with several other cytokeratin bands in addition to the p56 antigen. These additional reactive proteins, present in both the cell lysate and cytoskeletal protein preparation, do not seem to cross-link to the DNA, either with *cis*-DDP or with K_2CrO_4 (panels D and E, lanes 1 and 4, respectively). Conceivably, some may represent enzymatic degradation products of the cytokeratins p56 and p49; however, with recent two-dimensional gels and immunoblots,[2] we have identified these un-cross-linked species at cytokeratins B, C, E, and F in the designation described by Franke et al. (1981a).

With the saturation cross-linking experiment illustrated in Figure 2D, an estimate was made of the total cellular p39, p49,

[2] W. N. Schmidt and L. S. Hnilica, unpublished results.

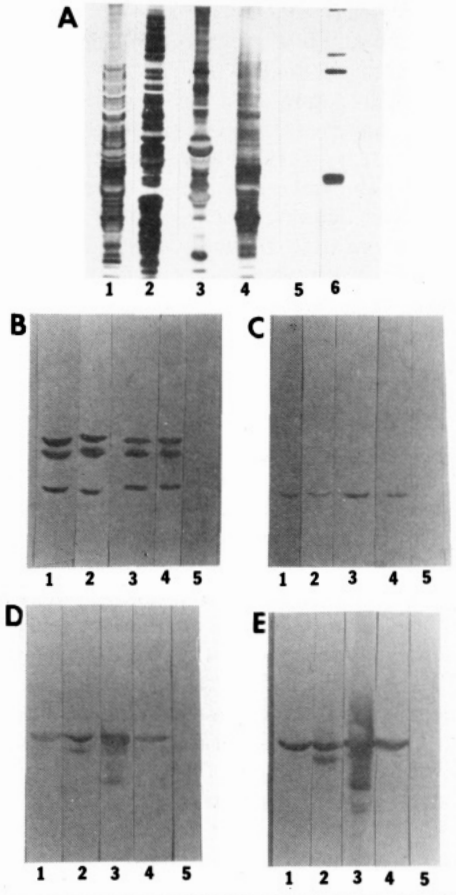

FIGURE 4: Comparison of cytokeratin–DNA cross-linking by K_2CrO_4 and *cis*-DDP. Panel A represents the SDS–polyacrylamide gel stained with $AgNO_3$ depicting similarities and differences of proteins in the high-speed DNA pellets of NAH cells incubated with 1 mM K_2CrO_4 or *cis*-DDP for 8 h. Lane 1, 1 mM *cis*-DDP cells; lane 2, cell lysate loaded at 1×10^5 cells/lane; lane 3, cytoskeletal protein enriched preparation; lane 4, 1 mM K_2CrO_4 cells; lane 5, control cells incubated for 8 h in the absence of cross-linker; lane 6, molecular weight standards as in Figure 1. Lanes 1, 4, and 5 loaded 10 μg/well as DNA. Lanes 2 and 3 loaded 5 μg of protein/well. The gel was purposely overstained to check for the possible presence of cytokeratins in lane 5, as well as to make a comparison between the two metal cross-linkers by allowing many faint bands (which are invisible with short staining) to develop. Panels B–E represent selective cross-linking of cytokeratins to DNA in high-speed DNA pellets obtained from cells incubated with 1 mM *cis*-DDP or K_2CrO_4 for 8 h; immunoblots incubated with four different antibodies are shown. Lanes 1–5 in panels B–E contain duplicate samples as indicated in panel A. (B) Polyclonal antibody to NAH cytokeratin. (C) Monoclonal antibody to p39. (D) Monoclonal antibody C7 to cytokeratin p56. (E) Monoclonal antibody B9 to cytokeratin p56.

Table II: Comparison of p56, p49, and p39 Ratios in Various Cellular Fractions and Cross-Linked DNA Pellets

source of material	cytokeratin cross-linked (%)[a]		
	p56	p49	p39
whole cell lysate[b]	59	22	21
cytoskeletal preparation[b]	34	36	28
0.5 mM K_2CrO_4	48	40	12
1.0 mM K_2CrO_4	45	29	26
1.0 mM *cis*-DDP	44	29	27

[a] The sum of the three cross-linked cytokeratins was assumed to be 100% after 8 h of incubation. [b] Not cross-linked.

and p56 cytokeratin that became cross-linked to the DNA in 3 mM K_2CrO_4. Assuming the amount of DNA in each NAH cell to be 6 pg and knowing the number of cells corresponding to a given volume of cell lysate, the cellular concentrations of the p39, p49, and p56 cytokeratins were calculated from

densitometric scans and are presented in Table II as percentages of the sum of these three cytokeratins. As can be seen, isolation of cytoskeletal proteins changed their relative ratios as compared to those of the cellular lysate, which can be assumed to represent the in vivo situation. Exposure of cells to either K_2CrO_4 or *cis*-DDP altered these ratios, showing that the p39 and p49 cytokeratins became preferentially cross-linked to DNA. However, there appears little, if any, difference in the relative amounts of the three cytokeratins cross-linked by either of the two metals as the cross-linking began to approach saturation at 1 mM K_2CrO_4 of *cis*-DDP.

The cross-linking of cytokeratins with DNA after the incubation of NAH cells with 1 mM K_2CrO_4 for 8 h was further confirmed by equilibrium centrifugation in CsCl density gradients (Figure 5A). Because of the small amounts of DNA-associated proteins, corresponding fractions (0.5 mL) from six parallel gradients were combined. The single 0.5-mL fraction on top of the gradient contained aggregated proteins in particulate form and was not, therefore, mixed with the other fractions. The pools were then electrophoresed, transferred to nitrocellulose sheets, and reacted with appropriate antisera. Densitometric scans of the electrophoretograms reacted separately with antisera to p39 and p56 are shown in Figure 5B. Only the top fractions (insoluble "skin" on top of the gradient at the average density 1.42 g/cm^3) and fractions 13–16 at the average density 1.62 g/cm^3 contained proteins reactive with this antiserum. The free DNA peak was at average density 1.71 g/cm^3 in fractions 17–22. Fractions 23–26, with OD 260 even higher than those of fractions 17–22, most likely contained RNA but no cytokeratin antigens.

To demonstrate the cytokeratin association with DNA, the cross-linked, pelleted DNA was first digested with micrococcal nuclease (100 units/mL) and DNase I (100 μg/mL) at 20 °C for 1 h and then centrifuged in CsCl gradient. The densitometric scans of immunoblots representing the pooled fractions are shown in Figure 5C. Only the fraction pool 2–4 (average density 1.45 g/cm^3) contained the p56 cytokeratin antigen (dotted line), while undigested control localized the same antigen (solid line) in the fraction pool 13–16 (average density 1.63 g/cm^3). Similar results were obtained with antibodies to cytokeratins p39 and p49. Thus, digestion on the DNA resulted in release of the cross-linked cytokeratins to the top of CsCl gradient.

Discussion

It is well-known that heavy metals can enter the cell nucleus and react with the cellular genome [reviewed by Heck & Costa (1982a,b) and Sunderman (1984)]. In addition to DNA damage, extensive DNA–protein cross-linking has been reported to occur in cells exposed to various compounds of Ni, Cr, or Pt (Ciccarelli et al., 1981; Ciccarelli & Wetterhahn, 1982, 1984, 1985; Lee et al., 1982; Fornace et al., 1981; Tsapakos et al., 1981, 1983; Robinson & Costa, 1982; Cupo & Wetterhahn, 1984; Wedrychowski et al., 1985; Zwelling et al., 1979a,b; Lippard & Hoeschele, 1979; Filipski et al., 1983; Banjar et al., 1984; Ward et al., 1984). With only a few exceptions the evidence for DNA–protein cross-link formation has been obtained through alkaline elution. This procedure, although quantitative, does not provide information about the nature of the cross-linked proteins (Kohn et al., 1981). We have shown that electrophoretic separation of the cross-linked protein species followed by their identification by specific antibodies offers a sensitive tool for detailed analysis of the heavy-metal interactions with nuclear components.

Although it is generally accepted that the protein families involved in the formation of intermediate filaments are im-

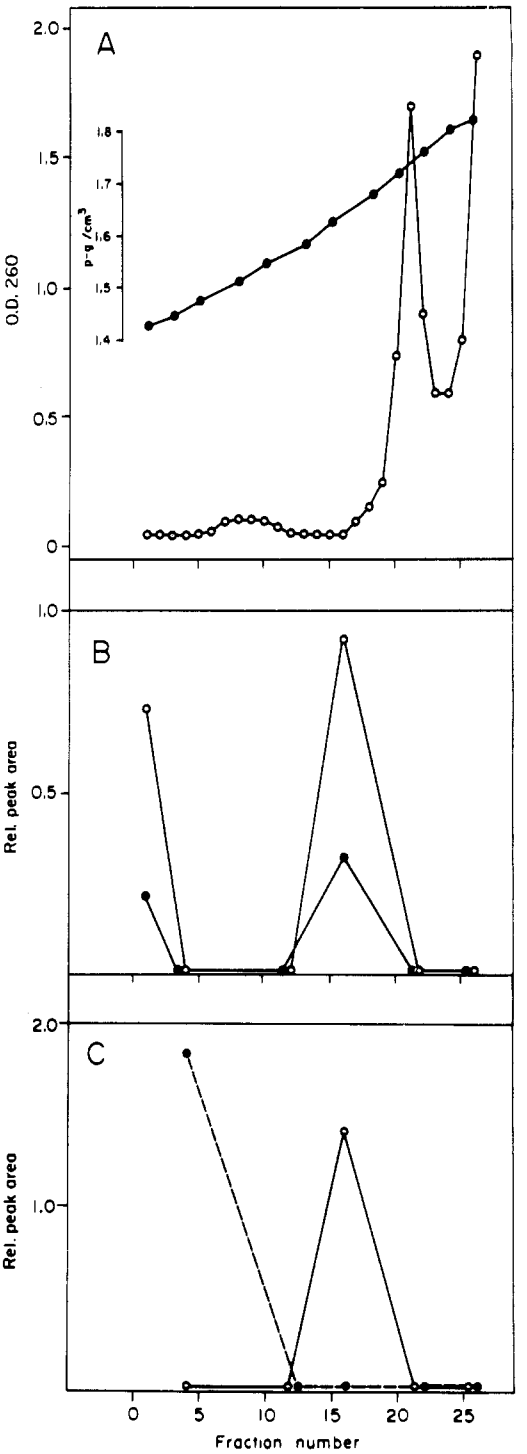

FIGURE 5: CsCl equilibrium density gradient centrifugation of high-speed DNA pellet fractions from NAH cells incubated with 1 mM K_2CrO_4 for 8 h. (A) The OD at 260 nm (○) and density in g/cm^3 (●) of the 0.5-mL fractions collected from the gradient are shown. (B) Corresponding fractions from six identical gradients were pooled as described under Materials and Methods to make a total of six pools. The six pools were dialyzed, treated with DNase I, lyophilized, electrophoresed, transferred to nitrocellulose, and reacted separately with monoclonal antibodies reactive with NAH p39 and p56. The relative amounts of cytokeratins in each pool were determined by densitometric scanning of each of the six lanes of the nitrocellulose transfers. ●, cytokeratin p39; ○, cytokeratin p56. (C) In a separate experiment the DNA pellet was first digested with micrococcal nuclease (100 units/mL) and DNase I (100 μg/mL) at 20 °C for 1 h and then centrifuged in CsCl density gradient to an equilibrium. Five pools were collected (pool 1 representing the insoluble "skin" at the top of the gradient was discarded for clarity) and processed as in panel B. Only the reaction of monoclonal antibody to p56 cytokeratin is shown in this figure. ●, cytokeratin p56 in the nuclease-digested pellet; ○, the same cytokeratin in the undigested control.

portant for maintaining the structural integrity of eukaryotic cells, their exact biological function is not known. Cell-type specificity, allowing classification of cells on the basis of their intermediate filament protein expression, suggests that these proteins may be involved in more than just maintenance of cellular shape and positioning of the cell nucleus (Osborn et al., 1981; Franke et al., 1981c). Subcellular fractionation experiments supported by electron microscopy and immunochemical location have shown the intimate association of intermediate filaments with the cell nucleus (Lehto et al., 1978; Small & Celis, 1978; Franke et al., 1979; Bennet et al., 1978; Goldman et al., 1979, 1983; Staufenbiel & Deppert, 1982; Capco & Penman, 1983; Steinert et al., 1984). It was suggested that the nuclear envelope may contain intermediate filament organizing centers (Eckert et al., 1981, 1982) that associate with the nuclear pore–lamina complex (Goldman et al., 1983; Steinert et al., 1984). Indeed, using whole-mount electron microscopy, Capco and colleagues (Capco et al., 1982; Capco & Penman, 1983) demonstrated the intimate relationship between the cytoplasmic filaments, the nuclear matrix, and the chromosomes of mitotic cells.

Cells exposed to antimitotic drugs (Goldman & Knipe, 1972; Gordon et al., 1978; Hynes & Destree, 1978; Starger et al., 1978) or microinjected with antibodies to intermediate filament proteins (Eckert et al., 1981, 1982; Lane & Klymkowsky, 1981; Lin & Feramisco, 1981) have their intermediate filaments collapse reversibly onto the nuclear surface. The anchorage of intermediate filaments to the cell nucleus is further supported by the experiments of Hedgecock & Thomson (1982), who produced single gene mutations in the nematode *Caenorhabditis elegans*. This gene, anc 1, most likely codes for one of the cytoskeletal proteins, and its mutation resulted in loss of nuclear anchorage and inappropriate positioning of desmosomes in the affected cells.

Using monoclonal antibody reactive with just one cytokeratin species, the Novikoff hepatoma p39 protein, Ward et al. (1984) presented evidence that a portion of the total amount of this antigen in the cell becomes cross-linked to the DNA when Novikoff hepatoma cells are incubated in 1 mM *cis*-DDP or exposed to γ radiation. Our results presented here are in agreement with this observation and extend it to other Novikoff hepatoma cytokeratins. Indeed, all the three principal cytokeratins forming intermediate filaments of this transplantable tumor became cross-linked when Novikoff hepatoma cells were incubated in the presence of K_2CrO_4 or *cis*-DDP.

The concentration-dependent increase of the cross-linked cytokeratins (reaching saturation above 2 mM concentration of K_2CrO_4) as detected with poly- or monoclonal antibodies to these proteins when the cells were incubated either for various time periods or with increasing concentrations of the metal indicates that the association of cytokeratins with the cell nucleus and their proximity to DNA are natural phenomena. A nonlinear increase with no saturability of the DNA binding sites should be expected if the cross-linking were the result of nonspecific aggregation and precipitation. This notion is further supported by the virtual absence of protein–protein cross-links at K_2CrO_4 concentrations of 1 mM and less. Such cross-links would be easily detectable on immunoblots of the electrophoretically separated proteins and can be actually seen in immunoblots of cross-linked proteins at K_2CrO_4 concentrations above 1 mM.

The lag in cross-linking at low K_2CrO_4 concentrations or during the first 2 h of incubation can be explained by the necessity of hexavalent chromium to be converted to a trivalent form. Although the former can easily cross cellular membranes, only the latter has been shown to react with DNA and form DNA–protein cross-links (Fornace et al., 1981). Living cells are impenetrable to trivalent chromium compounds. We have previously shown (Wedrychoswki et al., 1985) that when Novikoff hepatoma cells are incubated with [^{51}Cr]chromate, cellular uptake of the metal is highest during the first 4 h of incubation while nuclear accumulation begins only after 2 h and coincides with the rates of DNA–protein cross-link formation.

Both *cis*-DDP and K_2CrO_4 in equimolar concentrations cross-linked the same three major keratins. They failed, however, to cross-link all the cytokeratins in preparations of cytoskeletal proteins as detected by our antibodies (Figures 3A and 4D,E). These results indicate a differential ability of some cytokeratins to associate with DNA and may suggest possible regulatory sites for involvement of cytokeratins in cellular functions.

The p39 cytokeratin exhibited a different apparent rate of cross-link formation and concentration dependence for DNA cross-linking than the p56 and p49 cytokeratins. This observation may be explained by differences in affinities of the individual proteins for chromium, by cooperative cross-linking of the individual cytokeratins to DNA, or may reflect rearrangements of the cytoskeletal architecture perturbed by the presence of a heavy metal. Further information on the DNA and protein sequence specificity required for these interactions is needed for clarification of this phenomenon. The kinetic differences, however, likely reflect individual selectivities for these associations, which also may imply a heterogeneity of the role of the proteins in filament position, structure, and function.

On the basis of quantitative assumptions described under Results and with data from Table II, the amount of each of the three DNA-cross-linked cytokeratins can be calculated as a fraction of total cellular p39, p49, and p56 concentration. These values for Novikoff hepatoma cells are 10.9%, 5.5%, and 4.3%, respectively. This indicates that only a small fraction of the cellular intermediate filament proteins associates with the cell nucleus and can be cross-linked to the DNA. At this time, the reasons for the differences in the amount of each protein that can be cross-linked to DNA are not known. These values, however, are compatible with our earlier suggestion that only the filament ends may be near enough or bound to DNA to become cross-linked (Ward et al., 1984).

ACKNOWLEDGMENTS

We thank Doris Harris for her outstanding assistance in the preparation of the manuscript.

REFERENCES

Bakke, O., Jakobsen, K., & Eik-Nes, K. B. (1984) *Cytometry 5*, 482–486.

Banjar, Z. M., Hnilica, L. S., Briggs, R. C., Stein, J., & Stein, G. (1984) *Biochemistry 23*, 1921–1926.

Bannasch, P., Zerban, H., Schmid, E., & Franke, W. W. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4948–4952.

Battifora, H., Sun, T. T., Bahu, R. M., & Rao, S. (1980) *Hum. Pathol. 11*, 635–641.

Bennet, G. S., Fellini, S. A., Croop, J. M., Otto, J. J., Bryan, J., & Holtzer, H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4364–4368.

Bradley, M. O., Patterson, S., & Zwelling, L. A. (1982) *Mutat. Res. 96*, 67–74.

Capco, D. G., & Penman, S. (1983) *J. Cell Biol. 96*, 896–906.

Capco, D. G., Wan, K. M., & Penman, S. (1982) *Cell (Cambridge, Mass.) 29*, 847–858.

Ciccarelli, R. B., & Wetterhahn, K. E. (1982) *Cancer Res. 42*, 3544–3549.

Ciccarelli, R. B., & Wetterhahn, K. E. (1984) *Cancer Res. 44*, 3892–3897.

Ciccarelli, R. B., & Wetterhahn, K. E. (1985) *Chem.-Biol. Interact. 52*, 347–360.

Ciccarelli, R. B., Hampton, T. H., & Jennette, K. W. (1981) *Cancer Lett. (Shannon, Irel.) 12*, 349–354.

Cupo, D. Y., & Wetterhahn, K. E. (1984) *Carcinogenesis (London) 5*, 1705–1708.

Eckert, B. S., Daley, R. A., & Parysek, L. M. (1981) *Cold Spring Harbor Symp. Quant. Biol. 46*, 403–412.

Eckert, B. S., Daley, B. A., & Parysek, L. M. (1982) *J. Cell Biol. 92*, 575–578.

Filipski, J., Kohn, K. W., & Bonner, W. M. (1983) *FEBS Lett. 152*, 105–108.

Fornace, A. J., Seres, D. S., Lechner, J. F., & Harris, C. C. (1981) *Chem.-Biol. Interact. 36*, 345–354.

Franke, W. W., Schmid, E., Weber, K., & Osborn, N. (1979) *Exp. Cell Res. 118*, 95–109.

Franke, W. W., Denk, H., Kalt, R., & Schmid, E. (1981a) *Exp. Cell Res. 131*, 299–318.

Franke, W. W., Schiller, D. L., Moll, R., Winter, S., Schmid, E., Engelbrecht, I., Denk, H., Krepler, R., & Platzer, B. (1981b) *J. Mol. Biol. 153*, 933–959.

Franke, W. W., Schmid, E., Schiller, D. L., Winter, S., Jarasch, E. D., Moll, R., Denk, H., Jackson, B. W., & Illmensee, K. (1981c) *Cold Spring Harbor Symp. Quant. Biol. 46*, 431–453.

Fuchs, E., Grace, M. P., Kim, K. H., & Marchuk, D. (1984) in *Cancer Cells: 1. The Transformed Phenotype* (Levine, A. J., Vande Woude, G. F., Topp, W. C., & Watson, J. D., Eds.) pp 161–167, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Fujitani, H., Chiu, J., & Hnilica, L. S. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1943–1946.

Geiger, B., Kreis, T. E., Gigi, O., Schmid, E., Mittnacht, S., Jorcano, J. L., von Bassewitz, D. B., & Franke, W. W. (1984) in *Cancer Cells: 1. The Transformed Phenotype* (Levine, A. J., Vande Woude, G. F., Topp, W. C., & Watson, J. D., Eds.) pp 201–215, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Goldman, R. D., & Knipe, D. M. (1972) *Cold Spring Harbor Symp. Quant. Biol. 37*, 523–533.

Goldman, R. D., Milsted, A., Schloss, J. A., Starger, J., & Yerna, M.-J. (1979) *Annu. Rev. Physiol. 41*, 703–722.

Goldman, R. D., Goldman, A. E., Jones, J., Maul, G., Yang, H., & Zackroff, R. (1983) *J. Cell Biol. 97*, 221a.

Gordon, W. E., Bushnell, A., & Burridge, K. (1978) *Cell (Cambridge, Mass.) 13*, 249–261.

Guengerich, F. P., Wang, P., & Davidson, N. K. (1982) *Biochemistry 21*, 1699–1706.

Heck, J. D., & Costa, M. (1982a) *Biol. Trace Elem. Res. 4*, 71–82.

Heck, J. D., & Costa, M. (1982b) *Biol. Trace Elem. Res. 4*, 319–330.

Hedgecock, E. M., & Thomson, J. N. (1982) *Cell (Cambridge, Mass.) 30*, 321–330.

Hynes, R. O., & Destree, A. T. (1978) *Cell (Cambridge, Mass.) 13*, 151–163.

Kohn, K. W., Ewig, R. A. G., Erickson, L. C., & Zwelling, L. A. (1981) in *DNA Repair. A Laboratory Manual of Research Procedures* (Freidberg, E. C., & Hanawalt, P. C., Eds.) Vol. I, Part B, pp 379–401, Marcel Dekker, New York.

Köpf-Maier, P., Wagner, W., & Liss, E. (1983) *J. Cancer Res. Clin. Oncol. 106*, 44–52.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lane, E. B., & Klymkowsky, M. W. (1981) *Cold Spring Harbor Symp. Quant. Biol. 46*, 387–402.

Lazarides, E. (1982) *Annu. Rev. Biochem. 51*, 219–250.

Lee, J. E., Ciccarelli, R. B., & Jennette, K. W. (1982) *Biochemistry 21*, 771–778.

Lehto, V., Virtanen, I., & Kurki, P. (1978) *Nature (London) 272*, 175–177.

Lin, J. J.-C., & Feramisco, J. R. (1981) *Cell (Cambridge, Mass.) 24*, 185–193.

Lippard, S. J., & Hoeschele, J. D. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6091–6095.

Ochs, D. (1983) *Anal. Biochem. 135*, 470–474.

Osborn, M., Geisler, N., Shaw, C., Sharp, G., & Weber, K. (1981) *Cold Spring Harbor Symp. Quant. Biol. 46*, 413–429.

Rheinwald, J. G., O'Connell, T. M., Connell, N. D., Rybak, S. M., Allen-Hoffmann, B. L., La Rocca, P. J., Wu, Y. J., & Rehwoldt, S. M. (1984) in *Cancer Cells: 1. The Transformed Phenotype* (Levine, A. J., Vande Woude, G. F., Topp, W. C., & Watson, J. D., Eds.) pp 217–227, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Robinson, S. H., & Costa, M. (1982) *Cancer Lett. (Shannon, Irel.) 15*, 35–40.

Sanderson, C. J. (1976a) *Proc. R. Soc. London, B 192*, 221–239.

Sanderson, C. J. (1976b) *Transplantation 21*, 526–529.

Schlegel, R., Banks-Schlegel, S., McLeod, J. A., & Pinkus, G. S. (1980) *Am. J. Pathol. 110*, 41–49.

Schmidt, W. N., & Hnilica, L. S. (1982) *Cancer Res. 42*, 1441–1445.

Schmidt, W. N., Stryjecka-Zimmer, M., Glass, W. F., II, Briggs, R. C., & Hnilica, L. S. (1981) *J. Biol. Chem. 256*, 8117–8123.

Schmidt, W. N., Pardue, R. L., Tutt, M. C., Briggs, R. C., Brinkley, B. R., & Hnilica, L. S. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3138–3142.

Schmidt, W. N., Page, D. L., Schmidt, C. A., McKusick, K. B., Ward, S. W., & Hnilica, L. S. (1984) *Cancer Res. 44*, 5867–5879.

Small, J. V., & Celis, J. E. (1978) *J. Cell. Sci. 31*, 393–409.

Starger, S. M., Brown, W. E., Goldman, A. E., & Goldman, R. D. (1978) *J. Cell Biol. 78*, 93–109.

Staufenbiel, M., & Deppert, W. (1982) *Exp. Cell Res. 138*, 207–214.

Steinert, P. M., Jones, J. C. R., & Goldman, R. D. (1984) *J. Cell Biol. 99*, 22s–27s.

Sternberger, L. A. (1979) *Immunochemistry*, 2nd ed., pp 122–129, Wiley, New York.

Sun, T. T., Eichner, R., Schermer, A., Cooper, D., Nelson, W. G., & Weiss, R. A. (1984) in *Cancer Cells: 1. The Transformed Phenotype* (Levine, A. J., Vande Woude, G. F., Topp, W. C., & Watson, J. D., Eds.) pp 169–176, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sunderman, W. F., Jr. (1984) *Ann. Clin. Lab. Sci. 14*, 93–122.

Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1350–1354.

Tsapakos, M. J., Hampton, T. H., & Wetterhahn-Jennette, K. W. (1981) *J. Biol. Chem. 256*, 3623–3626.

Tsapakos, M. J., Hampton, T. H., Sinclair, P. R., Sinclair, J. F., Bement, W. J., & Wetterhahn, K. E. (1983) *Carcinogenesis (London) 4*, 959–966.

Ward, W. S., Schmidt, W. N., Schmidt, C. A., & Hnilica, L. S. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 419–423.
Ward, W. S., Schmidt, W. N., Schmidt, C. A., & Hnilica, L. S. (1985) *Biochemistry 24*, 4429–4434.
Weber, K., & Geisler, N. (1984) in *Cancer Cells: 1. The Transformed Phenotype* (Levine, A. J., Vande Woude, G. F., Topp, W. C., & Watson, J. D., Eds.) pp 153–159, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Wedrychowski, A., Ward, W. S., Schmidt, W. N., & Hnilica, L. S. (1985) *J. Biol. Chem. 260*, 7150–7155.
Wray, W., Boulikas, T., Wray, V. P., & Hancock, R. (1981) *Anal. Biochem. 118*, 179–203.
Zwelling, L. A., Anderson, T., & Kohn, K. W. (1979a) *Cancer Res. 39*, 365–369.
Zwelling, L. A., Bradley, M. O., Sharkey, N. A., Anderson, T., & Kohn, K. W. (1979b) *Mutat. Res. 67*, 211–280.

Isolation of Intercalator-Dependent Protein-Linked DNA Strand Cleavage Activity from Cell Nuclei and Identification as Topoisomerase II

Jon Minford, Yves Pommier, Jan Filipski,[‡] Kurt W. Kohn,* Donna Kerrigan, Michael Mattern, Steven Michaels, Ronald Schwartz, and Leonard A. Zwelling[§]

Laboratory of Molecular Pharmacology, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland 20205

Received March 29, 1985

ABSTRACT: DNA intercalating agents such as 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) have previously been found to induce in mammalian cells the formation of protein-associated DNA single- and double-strand breaks. In the current work, an activity characterized by the production of DNA–protein links associated with DNA strand breaks and by stimulation by *m*-AMSA was isolated from L1210 cell nuclei and was shown to be due to topoisomerase II. Nuclei were extracted with 0.35 M NaCl, and the extract was fractionated by gel filtration, DNA–cellulose chromatography, and glycerol gradient centrifugation. A rapid filter binding assay was devised to monitor the fractionation procedure on the basis of DNA–protein linking activity. The active DNA–cellulose fraction contained both topoisomerase I and topoisomerase II whereas the glycerol gradient purified material contained only topoisomerase II activity. The properties of the active material were studied at both stages of purification. *m*-AMSA enhanced the formation of complexes between purified topoisomerase II and SV40 DNA in which the DNA sustained a single- or double-strand cut and the enzyme was covalently linked to the 5′ terminus of the DNA. This action was further enhanced by ATP, as well as by nonhydrolyzable ATP analogues. *m*-AMSA inhibited the topoisomerization and catenation reactions of topoisomerase II, probably because of trapping of the enzyme–DNA complexes. The activity showed a dependence on the type of DNA intercalators used, analogous to what was previously observed in intact cells. *m*-AMSA had no effect on topoisomerase I. The results serve as a basis for the utilization of alkaline elution assays of drug-induced protein-associated DNA strand breaks as a functional measure of topoisomerase II in cells. *m*-AMSA enhanced the formation of DNA strand breaks and DNA–protein links optimally when the concentration of purified extract was low; at high extract concentrations, DNA strand breaks and DNA–protein links occurred even in the absence of *m*-AMSA. The possibility is discussed that DNA binding activity and topoisomerase activity are alternative functional states of the enzyme.

When mammalian cells are treated with certain DNA intercalating agents, such as ellipticine and 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA), DNA strand breaks can be detected which appear to be associated with DNA–protein cross-links (Ross et al., 1978, 1979; Zwelling et al., 1981). Ross et al. (1979) proposed that this effect is due to the trapping of a topoisomerase in a covalent DNA-bound state. The formation of protein-associated DNA strand breaks in cells treated with *m*-AMSA was found to be strongly temperature dependent and to saturate at about 60 000 per cell (Zwelling et al., 1981). Strand breaks of the single-strand and double-strand types occurred at ratios that differed for different intercalating compounds (Zwelling et al., 1982). It was originally proposed that the single- and double-strand breaks were produced by topoisomerase types I and II, stimulated to different degrees by different intercalators (Zwelling et al., 1981).

Filipski & Kohn (1982) demonstrated the formation of intercalator-induced protein-associated DNA strand breaks in isolated mouse L1210 cell nuclei. The reaction was reversible and required Mg^{2+}; it did not require the addition of ATP and was in fact stimulated by nonhydrolyzable ATP analogues (Pommier et al., 1982, 1984a). Filipski et al. (1983) utilized the isolated nuclei system to develop an assay for the responsible enzyme. The endogenous activity in isolated nuclei was found to be removed by extraction with 0.35 M NaCl. The intercalator-dependent formation of DNA strand breaks and DNA–protein cross-links (in one to one proportion) could then be restored by addition of 0.35 M NaCl nuclear extract. The extract was fractionated by gel filtration, and the activity

* Correspondence should be addressed to this author.
‡ Present address: Université Paris VII, Paris, France.
§ Present address: Department of Chemotherapy Research and Medical Oncology, Division of Medicine, M. D. Anderson Hospital, Houston, TX 77030.