Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 419–423, January 1984
Biochemistry

Association of cytokeratin p39 with DNA in intact Novikoff hepatoma cells

(*cis*-dichlorodiammineplatinum/intermediate filament/tumor-specific antigen)

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Communicated by Sidney P. Colowick, October 5, 1983

ABSTRACT **The relationship between the rat tumor-associated cytokeratin p39 (M_r 39,000) and cellular DNA has been studied in intact cells. Using a DNA–protein crosslinking technique, incubation with *cis*-dichlorodiammineplatinum, we present evidence for the association of p39 with DNA in intact Novikoff ascites hepatoma cells. The cells were treated with *cis*-dichlorodiammineplatinum and solubilized in NaDodSO$_4$-containing buffer, and the DNA was pelleted by high-speed centrifugation. By immunotransfer analysis, the cytokeratin was found in the DNA pellet of the crosslinked samples while absent from the controls. This result was further substantiated by CsCl density-gradient centrifugation. Collectively, these results suggest a cytokeratin–DNA association at the filament binding sites on or near the nuclear lamina.**

The cytokeratins are perhaps the most heterogeneous class of polypeptides that comprise the cytostructural intermediate filaments (1). These proteins exhibit overall patterns of cell-type specificity, allowing classification of epithelial and carcinoma cells on the basis of cytokeratin expression (2–5). Immunohistochemical experiments demonstrate keratin filaments to extend from desmosomal junctions to a supposed filament "organizing center" at the nucleus (6, 7). These attachment sites on or near the peripheral lamina of the nucleus are as yet undefined, and experiments attempting to assign a role for filaments (if any) in nuclear morphology and function are further complicated by the extreme insolubility of these species and their tenacious ability to remain with purified nuclei (8).

The three Novikoff ascites hepatoma (NAH) antigens p39, p49, and p56 (M_rs 39,000, 49,000, and 56,000, respectively) were originally characterized in chromatin preparations (9–11). Subsequent studies established the specificity of the p39 and p49 antigens for rat carcinoma cells (12, 13) and their identification as cytokeratins (14). Our laboratory has been concerned with clarifying the relationship of these antigens to the nucleus and chromosomal proteins. In this investigation, we have employed a protein–DNA crosslinker, *cis*-dichlorodiammineplatinum (*cis*-DDP), and γ irradiation with intact, viable cells to assess the *in vivo* interaction of the NAH p39 cytokeratin antigen with DNA. Our results indicate that a part of this cellular antigen, perhaps the filament ends, is situated either within crosslinking distance or bound to the DNA in the intact cell.

MATERIALS AND METHODS

NAH was maintained by weekly transplantation in male Sprague–Dawley rats. Electrophoresis supplies were purchased from Bio-Rad. DNase I was obtained from Worthington and density-gradient grade CsCl was from Gallard Schlesinger. Immunochemicals were from Miles and Sternberger-Meyer (Jarrettesville, MD). *cis*-DDP was obtained from Sigma.

Crosslinking with *cis*-DDP and γ Irradiation. Crosslinking experiments were performed essentially as described (15, 16), with modifications. For *cis*-DDP crosslinking, freshly collected NAH cells were gently washed with 5 vol of 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.2, and centrifuged at low speed (700 × *g*, 5 min). The cells were then resuspended in 20 vol of the same buffer with or without 2 mM *cis*-DDP and incubated for the indicated times at 37°C. Crosslinked cells were recovered by low-speed centrifugation and solubilized by homogenization and stirring in 20 vol of 4% NaDodSO$_4$/50 mM Tris·HCl, pH 7.5/1 mM phenylmethylsulfonyl fluoride, for 3–4 hr. After centrifugation of this suspension at 100,000 × *g* for 16 hr, the DNA-containing pellets were resuspended in the above NaDodSO$_4$ buffer and again centrifuged 16 hr at 100,000 × *g*. The final pellets were suspended in 2 mM Tris·HCl, pH 7.5/1 mM PhMeSO$_2$F and dialyzed overnight against the same buffer. Proteins associated with the DNA pellet were then assessed by immunotransfer methodology as described below.

For crosslinking with γ irradiation, freshly collected NAH was subjected to 100 krad (1 rad = 0.01 gray) of γ irradiation from a ^{137}Cs source. Controls were incubated at room temperature for the duration of the irradiation. Cells were then collected by low-speed centrifugation and processed as described above for *cis*-DDP crosslinking.

Isolation of Chromatin, Nuclei, and NAH Cytokeratin. NAH nuclei were purified from washed cells lysed by hypotonic shock (17). The method of Bonner *et al.* (18), as modified in our laboratory (19), was used to prepare chromatin from isolated nuclei, which included one washing in 0.3 M NaCl and resuspension in 2 mM Tris·HCl, pH 7.5/0.1 mM PhMeSO$_2$F. Aliquots of chromatin were stored at −20°C until use.

Cytokeratin from washed NAH cells was prepared exactly as described by Franke *et al.* (20), which included one cycle of solubilization in urea/2-mercaptoethanol buffer and precipitation with low-ionic strength buffer.

Monoclonal Antibodies. Detailed methods and characterization studies of mouse monoclonal antibodies to NAH p39 will appear elsewhere (unpublished data). In brief, BALB/c mice were immunized by four weekly injections with 100-μg samples of NAH cytokeratin in Freund's adjuvant and intraperitoneal booster injections 5 days before fusion. P3X63-Ag8.653 mouse myeloma cells (kindly supplied by Christopher Reading, University of Texas System Cancer Center, Houston, TX) were passed in Dulbecco's modified minimal essential medium and fused with freshly isolated, immunized spleen cells according to Pontecorvo *et al.* (21). After fusion, $\approx 5 \times 10^5$ cells were dispensed into each well of 96-well Costar plates and subjected to hypoxanthine/aminopterin/thymidine selection (22). Fourteen to 21 days later, wells were screened by ELISA with goat anti-mouse IgG–peroxidase conjugate (23). Positive wells were amplified and cloned at

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Abbreviations: NAH, Novikoff ascites hepatoma; PhMeSO$_2$F, phenylmethylsulfonyl fluoride; *cis*-DDP, *cis*-dichlorodiammineplatinum.

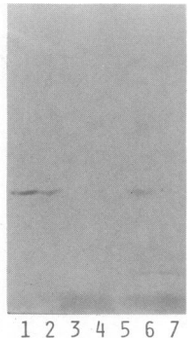

FIG. 1. Crosslinking of intact cells with *cis*-DDP. Freshly harvested NAH cells were crosslinked with *cis*-DDP and solubilized in $NaDodSO_4$-containing buffer, and high-speed DNA pellets were electrophoresed. Immunotransfer was incubated with monoclonal antibody to p39 and stained for immunoreactive proteins. Lane 1, normal NAH chromatin standard (25 μg as DNA); lanes 2–5, DNA pellets from cells incubated for 2 hr at 37°C with (lanes 2 and 4) or without (lanes 3 and 5) 2 mM *cis*-DDP and solubilized in 4% $NaDodSO_4$ with (lanes 4 and 5) or without (lanes 2 and 3) 5% (vol/vol) 2-mercaptoethanol (each lane is 300 μg as DNA); lane 6, DNA-pellet fraction from γ-irradiated cells (600 μg as DNA); and lane 7, uncrosslinked, control DNA pellet (600 μg as DNA).

least three times by limiting dilution assays using mouse thymocyte cells as a feeder layer and were assayed by immunotransfer methodology as described below. The hybridoma line G4 used in this investigation is a stable line producing IgM antibodies almost entirely specific for NAH antigen p39.

$NaDodSO_4$/PAGE and Immunoassays. High-speed DNA pellets were prepared for electrophoresis by DNase I shearing as described (12). Samples were electrophoresed under conditions of Laemmli (24), which employed a 3% stacking gel and a 7.5% resolving gel. Protein bands were either visualized with Coomassie brilliant blue staining or were transferred to nitrocellulose sheets as described by Towbin *et al.* (25). The method of Sternberger (26) was used to visualize immunoreactive antigens essentially as described (27). Immunoreactive staining was quantitated by densitometry with the method of Guengerich *et al.* (28).

CsCl Equilibrium Density-Gradient Ultracentrifugation. Control or treated cells were solubilized in $NaDodSO_4$ buffer and DNA was collected by ultracentrifugation, as described above. The DNA-containing pellets were resuspended in 2 mM Tris·HCl, pH 7.5/1 mM $PhMeSO_2F$, and ≈3 mg of DNA was gently homogenized in 42 ml of aqueous CsCl solution (1.60 g/ml). Ultracentrifugation was for 72 hr at 100,000 × g_{av} with a Beckman SW 40 rotor. Fractions of 0.5 ml were collected from the top of the gradient, measured for absorbance at 260 nm and density, pooled in groups of three or four, dialyzed overnight against 2 mM Tris·HCl, pH 7.5/0.1 mM $PhMeSO_2F$, lyophilized, and prepared for electrophoresis as described above.

RESULTS

Incubation of viable, intact NAH cells with *cis*-DDP resulted in the accumulation of the cytokeratin p39 into the high-speed DNA pellets of crosslinked, $NaDodSO_4$-solubilized cells, while the pellets of corresponding controls contained no p39 (Fig. 1). It should be emphasized that lanes 2–5 of Fig. 1 were loaded with samples adjusted to a DNA content 12-fold higher than that of the NAH chromatin standard on lane 1. Thus, only a small portion of the total p39 in chromatin was recovered in the DNA fraction after a 2-hr incubation

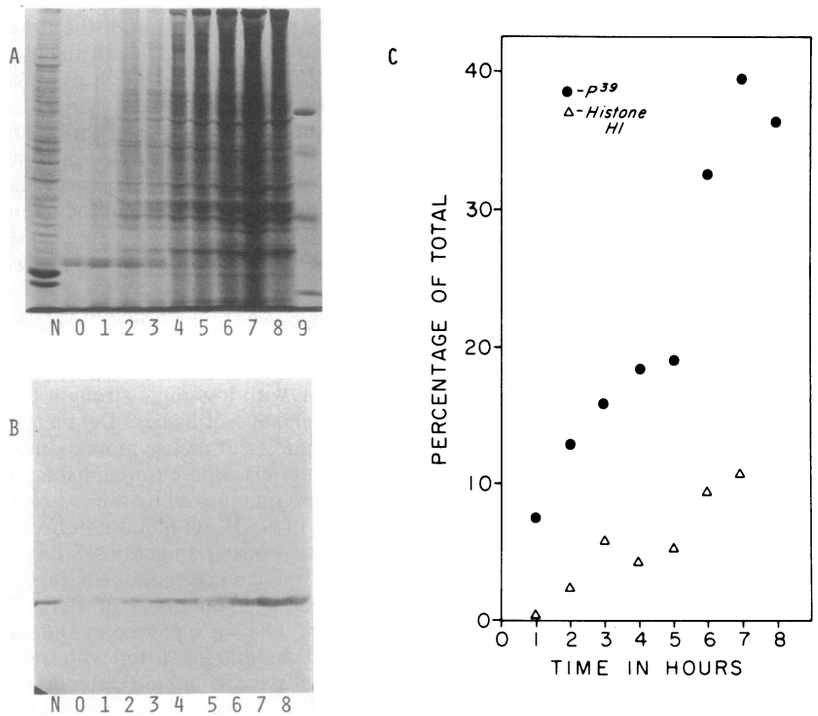

FIG. 2. Time course of the incorporation of p39 into the DNA pellets of *cis*-DDP-treated cells. Freshly harvested NAH cells were treated with 2 mM *cis*-DDP. At indicated intervals aliquots were solubilized in $NaDodSO_4$-containing buffer, and the DNA was pelleted by high-speed centrifugation, DNase I treated, subjected to $NaDodSO_4$/PAGE, and either stained with Coomassie brilliant blue (*A*) or transferred to nitrocellulose and reacted with monoclonal antibody to p39 (*B*). Lane N, normal Novikoff chromatin standard (25 μg as DNA); lanes 0–8, aliquots taken at 0–8 hr, respectively (150 μg as DNA); lane 9, molecular weight standards (Bio-Rad) (phosphorylase b, bovine serum albumin, ovalbumin, carbonic acid, and soybean trypsin inhibitor). (*C*) The amount of immunoreactive p39 was quantitated by densitometry (28), taking the amount of p39 in Novikoff chromatin as 100%. Histone H1 was quantitated by scanning the Coomassie-stained gel, taking the amount of H1 in chromatin as 100%.

with *cis*-DDP. When 5% (vol/vol) 2-mercaptoethanol was included in the solubilizing buffer, p39 was not recovered in the DNA pellet, suggesting that sulfydryl linkages may be important for the DNA–*cis*-DDP–p39 complex. This result was expected because sulfhydryl reducing agents have been shown to reverse DNA–*cis*-DDP–protein crosslinks (29). As a control, freshly harvested NAH cells were subjected to a large dose of γ irradiation, a technique known to crosslink DNA to protein (30, 31). p39 was found in the DNA pellet of irradiated samples (Fig. 1, lanes 6 and 7), though to a lesser extent than in *cis*-DDP treated samples (compare the DNA content in lanes 2 and 6, which contain roughly equivalent amounts of immunoreactive p39).

The effect of time on *cis*-DDP treatment was also tested. Freshly harvested NAH cells were incubated with *cis*-DDP and aliquots were removed at hourly intervals for 8 hr. The amount of immunoreactive p39 was assessed by densitometry in the immunotransfer profiles of the high-speed DNA pellets of $NaDodSO_4$-solubilized cell aliquots. With increasing time of *cis*-DDP treatment, we observed a nearly linear incorporation of p39 into the DNA pellet fractions up to about 7 hr; thereafter, the amount of p39 concentration did not increase (Fig. 2). The decreasing viability of cells past 8 hr in *cis*-DDP (about 80% of live cells, as assessed with trypan dye exclusion), precluded further time-course measurements. Assuming that the chromatin standard contained 100% of p39 available for DNA cross-linking, then our densitometry measurements indicated that about 40% of the chromosomal p39 associated with DNA after 7 hr of incubation. This amount of crosslinked p39 represents only about 10–20% of the total cellular p39 (10).

As a control we assessed the time-dependent appearance of a known DNA-binding chromosomal protein, H1 histone, into the DNA pellet fraction of *cis*-DDP-treated cells (Fig. 2). In this experiment, Coomassie-stained $NaDodSO_4$ gel profiles were scanned and peak areas corresponding to H1 histone were determined by integration. H1 histone became crosslinked to DNA and we noted an overall increase of H1 histone in the DNA pellets with extended incubation time. However, in contrast to p39, a smaller portion of the available H1 histone was crosslinked (about 10%) after 7 hr of incubation. These findings compare favorably with those of others showing only low or undetectable histone–DNA binding after *cis*-DDP treatment (32, 33).

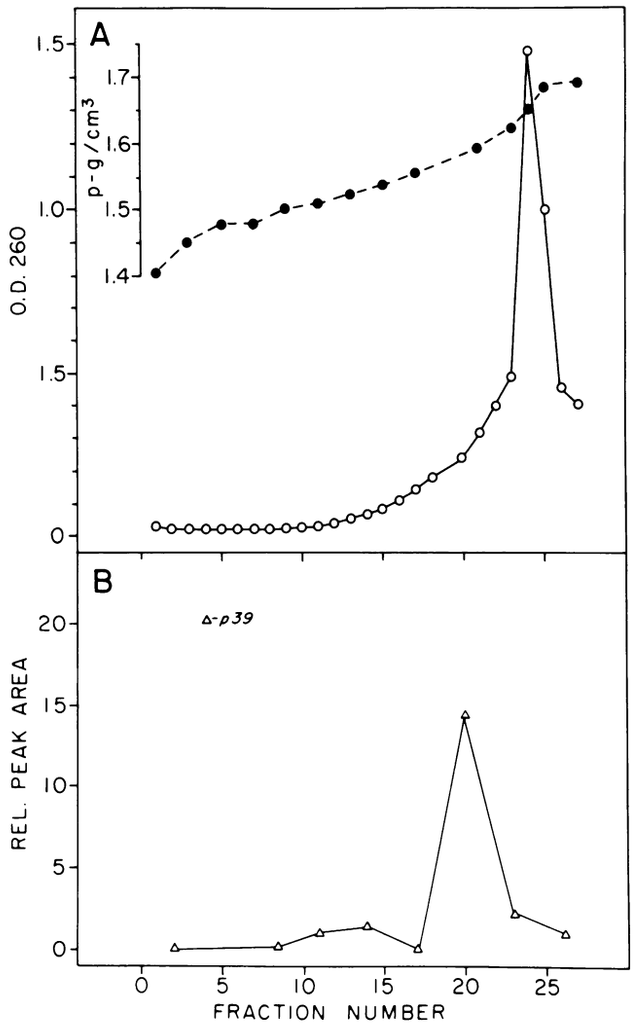

FIG. 3. CsCl equilibrium density-gradient centrifugation of high-speed DNA pellet fractions from whole cells reacted with *cis*-DDP for 2 hr. (*A*) The OD at 260 nm (○) and density in g/cm³ (●) of the fractions collected from gradients. (*B*) The fractions from three gradients were pooled according to OD at 260 to make a total of nine fractions. Each of the experimental points represents a pool of three or four fractions, 0.5 ml each. For this reason, the p39 antigenic peak is represented by only one point. However, these results were highly reproducible. These were then dialyzed, DNase I treated, lyophilized, electrophoresed, transferred to nitrocellulose, and reacted with monoclonal antibody to p39. The relative amount of p39 (△) for each fraction pool was then calculated as described.

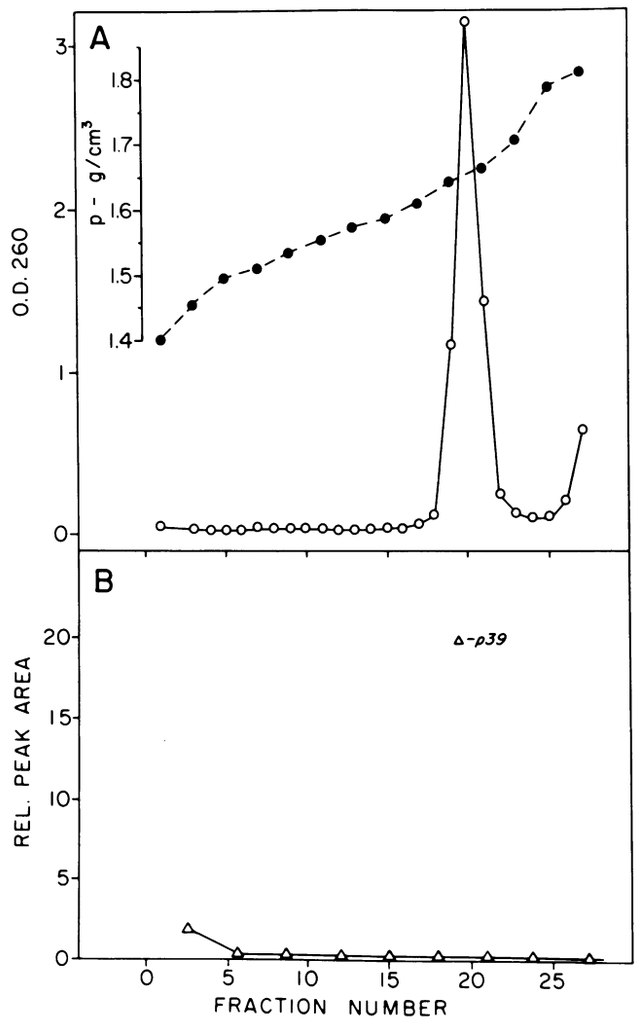

FIG. 4. CsCl equilibrium density-gradient centrifugation of $NaDodSO_4$-pelleted DNA of whole cells incubated at 37°C for 2 hr. (*A*) The OD at 260 nm (○) and density in g/cm³ (●) of the fractions collected from one gradient. (*B*) The fractions were treated exactly as described in the legend for Fig. 3.

To confirm the *cis*-DDP association of NAH p39 cytokeratin with DNA, experiments employing CsCl equilibrium density-gradient centrifugation were performed. High-speed DNA pellets from *cis*-DDP-treated or control cells were subjected to CsCl gradients, and the amount of immunoreactive cytokeratin antigen then was determined in pooled gradient fractions (Figs. 3 and 4). With *cis*-DDP crosslinking, p39 reactivity was observed to band at a density of almost 1.6 g/cm^3, which is greater than that of purified protein (1.3 g/cm^3) but less than that of pure DNA (1.7 g/cm^3). In contrast, gradients containing DNA pellets from uncrosslinked cells contained no detectable cytokeratin antigen within the gradient (Fig. 4). This result indicates that treatment of intact, viable NAH cells with protein–DNA crosslinkers results in a very tight, likely covalent, association of the p39 cytokeratin antigen with DNA.

DISCUSSION

Whether cytokeratins or other intermediate filaments are present in nuclear protein preparations because of their extreme insolubility or because of structural and functional relationships is an important question. We have obtained evidence indicating that a portion of one of the NAH cytokeratins, the p39 antigen, is situated in the intact cell within crosslinking distance of the DNA. Our experiments employed freshly harvested, viable cells and disruption directly in ionic detergent. These conditions eliminated the possibility of an artifactual cytokeratin–DNA binding complex.

Although *cis*-DDP has been documented to produce covalently bridged, protein–DNA complexes (32–35), the ability of the compound to elicit extensive DNA–DNA crosslinks raises the possibility that the cytokeratins may not directly bind to DNA but become trapped within a DNA cage during ultracentrifugation. Provided such a phenomenon occurs, it would still require considerable specificity because in our studies a known DNA binding protein, H1 histone, was crosslinked to DNA to a lesser extent than p39.

However, results obtained with the second protein–DNA crosslinking technique are not subject to this criticism because γ radiation causes double-stranded breaks in the DNA and no intrastrand DNA–DNA crosslinking (31). This may, in fact, be the reason that less p39 was recovered in the DNA pellets of γ-irradiated cells than in *cis*-DDP-treated cells because pelleting through $NaDodSO_4$ requires long DNA strands. However, regardless of the type of DNA–protein crosslinking agent, both experiments indicate that a portion of the cellular p39 is situated at, or near, DNA in intact NAH cells.

Parallel experiments with polyclonal antisera to whole NAH cytokeratin preparations (data not shown) indicated that all three principal NAH cytokeratins—p39, p49, and p56—can be crosslinked to cellular DNA. This suggests that cytokeratins and probably other intermediate filament proteins may actively participate in the nucleocytoplasmic interactions. The information obtained with monoclonal antisera to p39 shows that less than a half of the p39 present in chromatin becomes crosslinked with DNA. It should be further emphasized that the chromatin-associated p39 represents a rather small portion of its total cellular concentration (2). A possible explanation of these findings is that only the filament ends associate with the nuclear DNA on or near its interface with the nuclear membrane. In support of this hypothesis are recent high-voltage electron micrographs of whole cell mounts, illustrating the tight attachment of intermediate filaments to the nuclear lamina (36, 37), and our recent immunohistochemical experiments with monoclonal antibodies to p39, which show heavy perinuclear staining. Another possibility is the penetration of the nucleus by the cytokeratins with the filaments contributing to the fibrillar structure of the nuclear matrix (38, 39).

The overt neoplastic cell specificity of the p39 and p49 antigens (10, 12, 13) also raises the prospect that the NAH antigens may be unique in their association with cellular DNA (e.g., a situation only present in rat carcinoma cells). However, Traub *et al.* (40) have reported purified vimentin to have a high binding affinity for single-stranded DNA and RNA, which, in conjunction with our data, may indicate a more general relationship of the intermediate filaments and the cellular DNA.

This research was supported by National Institutes of Health Grant CA 26412 and by a fellowship from the Samuel Roberts Noble Foundation (W.S.W.).

1. Lazarides, E. (1982) *Annu. Rev. Biochem.* **51,** 219–250.
2. Battifora, H., Sun, T. T., Bahu, R. M. & Rao, S. (1980) *Hum. Pathol.* **11,** 635–641.
3. Bannasch, P., Zerban, H., Schmid, E. & Franke, W. W. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 4948–4952.
4. Schlegel, R., Banks-Schlegel, S., McLeod, J. A. & Pinkus, G. S. (1980) *Am. J. Pathol.* **110,** 41–49.
5. Franke, W. W., Schiller, D. L., Molle, R., Winter, S., Schmid, E., Engelbrecht, I., Denk, H., Krepler, R. & Platzer, B. (1981) *J. Mol. Biol.* **153,** 933–959.
6. Franke, W. W., Schmid, E., Weber, K. & Osborn, N. (1979) *Exp. Cell Res.* **118,** 95–109.
7. Eckert, B. S., Daley, R. A. & Parysek, L. M. (1982) *J. Cell. Biol.* **92,** 575–578.
8. Staufenbiel, M. & Deppert, W. (1982) *Exp. Cell Res.* **138,** 207–214.
9. Fujitani, H., Chiu, J. & Hnilica, L. S. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 1943–1946.
10. Schmidt, W. N., Stryjecka-Zimmer, M., Glass, W. F., II, Briggs, R. C. & Hnilica, L. S. (1981) *J. Biol. Chem.* **256,** 8117–8123.
11. Stryjecka-Zimmer, M., Schmidt, W. N., Briggs, R. C. & Hnilica, L. S. (1982) *Int. J. Biochem.* **14,** 591–600.
12. Schmidt, W. N. & Hnilica, L. S. (1982) *Cancer Res.* **42,** 1441–1445.
13. Schmidt, W. N., Gronert, B. J., McKusick, K. B., Page, D. L. & Hnilica, L. S. (1983) *Carcinogenesis* **4,** 675–681.
14. Schmidt, W. N., Pardue, R. L., Tutt, M. C., Briggs, R. C., Brinkely, B. R. & Hnilica, L. S. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 3138–3142.
15. Banjar, Z. M., Briggs, R. C., Hnilica, L. S., Stein, J. & Stein, G. (1983) *Biochem. Biophys. Res. Commun.* **114,** 767–773.
16. Banjar, Z. M., Briggs, R. C., Hnilica, L. S., Stein, J. & Stein, G. (1983) *Prog. Nucleic Acid Res. Mol. Biol.* **29,** 256–259.
17. Wilhelm, J. A., Ansevin, A. T., Johnson, A. W. & Hnilica, L. S. (1972) *Biochim. Biophys. Acta* **272,** 220–230.
18. Bonner, J., Chalkley, G. R., Dahmus, M., Fambrough, D., Fujimura, F., Huang, R. C., Huberman, J., Jensen, R., Marushige, K., Ohlenbusch, H., Oliviera, B. & Widholm, J. (1968) *Methods Enzymol.* **12,** 3–65.
19. Spelsberg, T. C. & Hnilica, L. S. (1972) *Biochim. Biophys. Acta* **228,** 202–211.
20. Franke, W. W., Weber, K., Osborn, M., Schmid, E. & Freudenstein, C. (1978) *Exp. Cell Res.* **116,** 429–445.
21. Pontecorvo, G. (1975) *Somatic Cell Genet.* **1,** 397–400.
22. Littlefield, J. W. (1964) *Science* **145,** 709.
23. Sanders, G. C. (1979) in *Immunoassays in the Clinical Laboratory*, eds. Nakamura, R. M., Dito, W. R. & Tucker, E. S., III (Liss, New York), pp. 99–118.
24. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
25. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 1350–1354.
26. Sternberger, L. A. (1974) *Immunochemistry* (Prentice-Hall, New York), pp. 129–171.
27. Glass, W. F., II, Briggs, R. C. & Hnilica, L. S. (1980) *Science* **211,** 70–72.
28. Guengerich, F. P., Wang, P. & Davidson, N. K. (1982) *Biochemistry* **21,** 1699–1706.
29. Filipski, J., Kohn, K. W., Prather, R. & Bonner, W. (1979) *Science* **204,** 181–183.
30. Olinski, R., Briggs, R. C. & Hnilica, L. S. (1981) *Radiat. Res.* **86,** 102–114.

31. Hawkins, R. B. (1979) *Int. J. Radiat. Biol.* **35,** 1–13.
32. Lippard, S. J. & Hoeschele, J. D. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 6091–6095.
33. Filipski, J., Kohn, K. W. & Bonner, W. M. (1983) *FEBS Lett.* **152,** 105–108.
34. Laurent, G., Erickson, L. C., Sharkey, N. A. & Kohn, K. W. (1981) *Cancer Res.* **41,** 3347–3351.
35. Ducore, J. M., Erickson, L. C., Zwelling, L. A., Laurent, G. & Kohn, K. W. (1982) *Cancer Res.* **42,** 897–902.
36. Capco, D. G., Wan, K. M. & Penman, S. (1982) *Cell* **29,** 847–858.
37. Capco, D. G. & Penman, S. (1983) *J. Cell Biol.* **96,** 896–906.
38. Kaufmann, S. H., Coffey, D. S. & Shaper, J. H. (1981) *Exp. Cell Res.* **132,** 105–123.
39. Berezney, R. (1980) *J. Cell Biol.* **85,** 641–650.
40. Traub, P., Nelson, W. J., Kuhn, S. & Vorgias, C. E. (1983) *J. Biol. Chem.* **258,** 1456–1466.