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The spatial distribution of exposed nuclear DNA in normal, cancer, and reverse-transformed cells

(chromatin/nuclease sensitivity/reverse transformation/cAMP/differentiation)

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ABSTRACT **The malignant CHO-K1 cell is reverse-transformed by cAMP, regaining the phenotype of a normal fibroblast. During this reaction, much of its DNA re-acquires sensitivity to hydrolysis by DNase I in a way characteristic of the normal fibroblast. Exposed DNA forms a rim about the nucleus in both the normal and reverse-transformed cell but not in the malignant CHO-K1. Reacquisition of the nuclear rim requires an organized cytoskeleton. Sequestered DNA forms families of different degrees of sequestration. In accordance with previous theoretical developments it is proposed that (*i*) genes specific to a given differentiation state are stored in the nuclear rim, whereas genes specific to other states are sequestered within the nucleus; (*ii*) only exposed genes are active, and their activity is modulated by regulatory molecules in the fluid medium; (*iii*) exposure and sequestration are regulated by cytoskeletal and nuclear protein structures; (*iv*) in at least several types of cancer the regulatory defect lies in the genome exposure process so that the specific DNA sequences and their associated growth regulatory loci have been transferred from the exposed to the sequestered condition with consequent loss of the nuclear rim of exposed DNA. The methodology described should be generally applicable to examining the accessibility state of subsets of DNA during various physiological modulations of cell function.**

Reverse transformation is a reaction in which malignant cells are restored to a normal phenotype in response to particular agonists as in the CHO-K1 cell treated with cAMP derivatives (1). Reverse transformation involves a succession of complex metabolic changes varying from early alterations in calcium ion dynamics occurring during the first minute (2) to changes in membrane and cytoskeletal structures (3, 4) and protein syntheses and phosphorylations (5–7) that extend over >48 hr. Ultimately, the pathway must result in specific changes in nuclear gene activity that restore specific differentiation and growth-control properties to the affected cells. We showed that an increase in DNA sensitivity to hydrolysis, which affects specific nuclear genes and involves a substantial fraction of the genome, occurs in reverse transformation (10, 11). This large-scale increase in DNase I sensitivity of chromatin after treatment of cells with specific reverse-transformation agents is now termed genome exposure. It was demonstrated that cytoskeletal integrity is required for this reaction and that the degree of increased exposure achieved in reverse transformation resembles that in normal cells (12).

Hutchison and Weintraub (9) showed that DNase I-sensitive regions of the genome in mouse L cells are preferentially localized at the nuclear periphery. The present paper describes experiments in which a modification of the techniques used by others (8, 9) is used to explore the nuclear location of exposed DNA in normal Chinese hamster ovary fibroblasts, the malignant CHO-K1, and in the latter cell after reverse transformation by cAMP reagents.

METHODS AND MATERIALS

Cells and Reagents. CHO-K1 is a subclone of the transformed Chinese hamster ovary cell isolated in this laboratory in 1958 (13). An early-passage line of the nonimmortalized CHO-784 cell was used as a normal fibroblast for comparison studies. Cells were grown in F-12 medium/7% fetal calf serum and maintained at 37°C in humidified 5% CO_2 atmosphere. For some experiments a defined medium (MCDB 302) supplemented with insulin and transferrin was used as described (11, 14).

The sources of special reagents were as follows: 8-bromoadenosine 3′,5′-cyclic monophosphoric acid (8BrcAMP) (Boehringer Mannheim); biotinylated nucleotides (Enzo Biochemicals); restriction enzymes, DNA polymerase I and Klenow fragment (Promega); DNase I (Bethesda Research Laboratories); antibodies; 4′,6,-diaminidino-2-phenylindole (DAPI), a fluorescent DNA-binding dye; and isobutylmethylxanthine (Sigma); streptavidin–alkaline phosphatase conjugate (Tago); and forskolin (Calbiochem).

***In Situ* Nick Translation.** Round glass coverslips (18 or 25 mm, no. 2 thickness) were precoated with poly(D-lysine) (30 min, 100 μg/ml) to increase adhesiveness. Approximately 3000 cells were seeded onto coverslips placed in 35-mm tissue culture dishes containing 2 ml of medium and were used for assay at the indicated times of growth. Coverslips were rinsed twice in phosphate-buffered saline and then fixed in cold methanol/acetic acid, 20:1, for 20 min, followed by rinses with phosphate-buffered saline to neutralize the pH (9). The reaction mixture was adapted from the reports of Kerem *et al.* (8) and Hutchison and Weintraub (9) to contain the following: 50 mM Tris·HCl, pH 7.9; 5 mM $MgCl_2$; 10 mM 2-mercaptoethanol; acetylated bovine serum albumin at 50 μg/ml; 4 units of *Escherichia coli* DNA polymerase I (or Klenow enzyme); 100 μM dATP, dCTP, dGTP, and 5 μM biotin-11-dUTP (as dTTP analogue). DNase was included in this mix at 1–33 ng/ml or omitted for control purposes. (One ng/ml is equivalent to ≈0.01 unit/ml.) The assay mixture was applied to cells by sandwiching 10 μl between the coverslip and a clean glass slide. Reactions occurred at room temperature and were stopped by immersing the coverslips in 20 mM EDTA solution, pH 8.0.

Our two standard detection systems for incorporated biotin were based on amplification reagents giving a colored reaction product as the final signal. Coverslips were washed in a buffer of moderate ionic strength (100 mM Tris·HCl, pH 7.5/150 mM NaCl/buffer 1) after the incorporation step and were then incubated for 30 min in buffer 1 containing acetyl-

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Abbreviations: 8BrcAMP, 8-bromoadenosine 3′,5′-cyclic monophosphoric acid; DAPI, 4′,6-diaminidino-2-phenylindole.

ated bovine serum albumin at 100 μg/ml and affinity reagents titrated to levels minimizing background binding. Goat anti-biotin antibody was used at 1:400 (15–20 μl per coverslip) followed by rabbit anti-goat IgG conjugated with alkaline phosphatase (1:500). Three washing steps with buffer were interposed between antibody incubations and color development. Alternatively, streptavidin–alkaline phosphatase conjugate was used at 1:10,000 as a direct biotin-binding ligand. In this case preincubation of the coverslip with unconjugated streptavidin (10 μg/ml) before the incorporation incubation was necessary to prevent a nonspecific adsorption to cytoplasmic structures. Coverslips with bound conjugates were rinsed briefly in alkaline buffer (100 mM Tris·HCl/100 mM NaCl/50 mM $MgCl_2$, pH 9.5), and the phosphatase reaction was developed in this buffer containing 40 mM of each nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Slides were monitored microscopically for the deposition of a purple reaction product at incorporation sites, and the reaction was stopped before over-development by immersion in stop buffer (10 mM Tris·HCl/5 mM EDTA, pH 8.0). Coverslips were air dried and mounted onto slides with Permount for evaluation and photomicroscopy.

Reverse transformation of CHO-K1 cells was affected by treatment with 0.5 mM 8BrcAMP or by 10 μM forskolin, a cAMP inducer, for 72 hr.

EXPERIMENTAL RESULTS

Comparison of Normal, Transformed, and Reverse-Transformed Cells. The *in situ* method described here for revealing the location of exposed DNA was applied to normal, transformed, and reverse-transformed Chinese hamster fibroblasts. Development of a color reaction with alkaline phosphatase conjugates specifically bound to incorporated biotinylated nucleotides marked the sites susceptible to nicking upon a brief treatment of fixed cells with exogenous DNase I. Typical results are presented in Fig. 1; (*i*) in Fig. 1*A* is shown the pattern produced by applying the *in situ* procedure to normal, early-passage Chinese hamster ovary fibroblasts (CHO-784). These cells, both in the presence and absence of 8BrcAMP or of forskolin, revealed the same pattern—i.e., the presence of the digestion-sensitive chromatin densely distributed about the nucleus periphery. The peripherally distributed exposed DNA was punctuated by regions of higher and lower density, and more sparse but definite exposed DNA regions were shown in the inner portions of the nucleus; (*ii*) untreated, malignant CHO-K1 cells showed only a sparse, patchy, or diffuse uptake of label that was not particularly associated with the nuclear periphery (Fig. 1*B*); (*iii*) if the cells in Fig. 1*B* were pretreated for 3 days with 8BrcAMP, however, a pattern of DNA exposure was demonstrated, consisting of a heavy band circling all or most of the nuclear periphery (Fig. 1*C*). As in untreated normal cells, the band was not completely uniform but at high magnification revealed the presence of punctate regions of higher density of the sensitive DNA. Minor amounts of labeled DNA also occurred in the nuclear interior and again showed discontinuities in degree of labeling. Similar results were obtained when isolated nuclei rather than whole cells were used for the nick-translation reaction. The similarity of results with fixed cells and with detergent-extracted nuclei prepared as in our earlier methods for electrophoretic gel analysis of DNA digestion (10–12) suggest that the bulk and the analytical methods measure aspects of the same phenomena of genome exposure. Fig. 1*D* presents a situation exactly like that in Fig. 1*C* except that DNase I treatment was omitted from the procedure. The absence of any visible deposit shows clearly that the effects obtained are due to the hydrolytic nicking of the DNA by the nuclease. The described differences in color development in the nuclear rim were consistently observed throughout the periods from early-to-late color formation during the reaction.

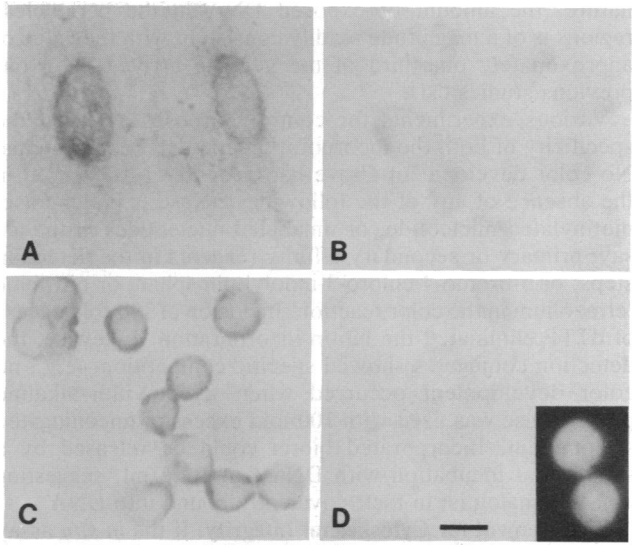

FIG. 1. Comparison of genome exposure in normal Chinese hamster ovary fibroblasts, transformed, and reverse-transformed CHO-K1 cells. Assays were done as described in text. (*A*) Normal, early-passage Chinese hamster fibroblasts. (*B*) Transformed CHO-K1 cells. (*C*) 8BrcAMP-treated CHO-K1. (*D*) 8BrcAMP-treated CHO-K1 except that no DNase I was included. (*Inset*) Typical appearance of CHO-K1 stained with DAPI, showing that DNA is distributed reasonably uniformly throughout the nucleus. (Bar = 10 μm.)

Fig. 1*D Inset* represents a picture of total DNA fluorescence obtained by treatment of CHO-K1 cells with fluorescent dye. Such cells whether treated or untreated with cAMP reveal a reasonably homogeneous distribution of DNA throughout the nucleus. Thus, the peripheral ring shown in the nuclei of cAMP-treated cells is not due to the existence of a pre-existing pattern in distribution of total DNA but rather only of DNA sensitive to DNase I hydrolysis. These results show that in reverse-transformed but not in untreated CHO-K1 cells, the location and extent of the pattern of enzyme-accessible chromatin approaches that characteristic of normal fibroblasts.

The difference in sensitive chromatin structure in response to 8BrcAMP treatment could often (14 of 22 comparisons) but not always be detected when the 8BrcAMP incubation period was reduced to 24 hr. Extending treatment time with 8BrcAMP from 3 to 7 days with or without subculturing did not enhance the labeling pattern. After 3-day treatment of cells with 8BrcAMP, further incubation for 24 hr in growth medium without cAMP caused the labeling profile to revert to the control pattern consistent with the previous observation of the phenotypic reversibility of the reverse-transformation phenomenon (1).

The effect of 8BrcAMP could be duplicated by treatment of cells with 10 μM forskolin plus 10^{-4} M isobutylmethylxanthine, an indication that the genome exposure is, indeed, responding to elevated intracellular cAMP levels. None of the following changes in conditions affected the difference in labeling between untreated and reverse-transformed CHO-K1 cells: growth on plastic rather than glass, use of defined serum-free medium rather than standard growth medium, using separate and optimized nicking and incorporation steps in the assays, carrying out the nick translation at 14°C, use of biotin-11-dCTP, or subculturing of cells 1 day before assay so that control and treated cells were present as single cells or small groups rather than as a contiguous population. Although the experiments reported here are qualitative in

nature, the amount of exposed DNA in the perinuclear regions is of a magnitude readily consistent with the value of approximately one-third of the genome arrived at in our previous studies (11).

Various experiments for control purposes assessed the specificity of both the incorporation and detection systems. No color development above background was observed in the absence of any of the following: DNase I, polymerase, biotinylated nucleotide, or unlabeled nucleotides in the assay; primary or secondary affinity reagents in the detection steps; or 5-bromo-4-chloro-3-indolyl phosphate or nitroblue tetrazolium in the color reaction. Inclusion of 100-fold excess of dTTP eliminated the biotin incorporation. Likewise, the detection conjugates showed specific competition—e.g., no color development occurred when streptavidin–alkaline phosphatase was used with 100-fold excess of unconjugated streptavidin. Incorporated biotin could be released by a subsequent incubation with DNase at 1 μg/ml, suggesting that the analog is, in fact, covalently bound into DNA.

Requirement for Cytoskeletal Integrity. If the *in situ* assay measures an aspect of the genome-exposure reaction previously defined by the gel analysis (12), it should also show the requirement for cytoskeletal integrity. Experiments were done in which the CHO-K1 cells grown for 72 hr in the presence of 8BrcAMP were treated with 2.6 μM colcemid for 2 1/2 hr before fixation for the *in situ* labeling assay. The presence of the colcemid largely eliminated the usual heavy labeling around the nuclear periphery seen in 8BrcAMP-treated cells (Fig. 2). Similar results were obtained by using 5 μM cytochalasin B for colcemid. In both cases the drug-treated cells showed a markedly diminished labeling of the nuclear periphery as well as decreased color intensity throughout the entire nucleus.

Association of DNase I-Sensitive Chromatin with the Insoluble Matrix. Nuclear matrix/cytoskeletal preparations were prepared by salt and detergent extraction of CHO-K1 cells grown on coverslips following the procedure of Capco *et al.* (15). When the nick-translation procedure is preceded by extraction with moderate concentrations of salt, the peripheral nuclear ring that differentiates the 8BrcAMP-treated from the untreated cell was left intact (Fig. 3 *A* and *B*). Use of a high salt extraction, however, which presumably removes many more protein constituents, leaving the nuclear matrix behind, still left a clear difference between the control and reverse-transformed cell, but the staining intensity of the latter was markedly diminished. Fig. 3*C* shows the result obtained from 8BrcAMP-treated cells. Control cells similarly treated were completely blank. These experiments suggest that some exposed DNA in the reverse-transformed cell is intimately connected with the nuclear matrix. Of considerable interest are two other features. A punctate pattern of exposed DNA covered the outermost lamina of high salt-extracted matrices. Moreover, there was a definite suggestion of a linear pattern connecting the punctate deposits. A physical association of labeled DNA with the lamin polymer system is suggested.

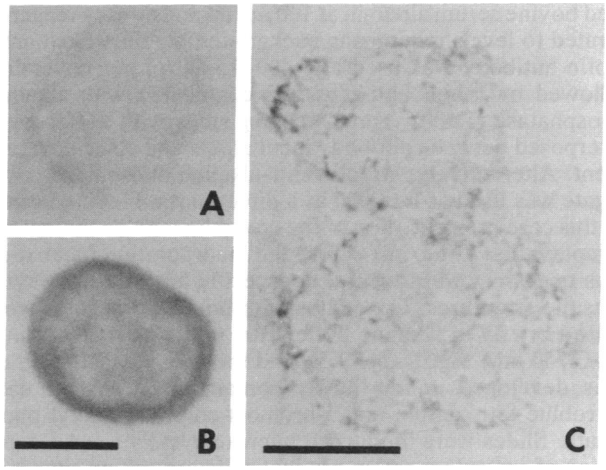

FIG. 3. Nuclear matrix localization of labeled chromatin. Matrices were prepared using the CSK (*A* and *B*) and CSK-AS (*C*) buffers of Capco *et al.* (15). Two nuclei are shown in *C*. *B* and *C* are 8BrcAMP treated, whereas *A* is untreated. The micrographs show the labeling pattern of 8BrcAMP-treated CHO-K1. Matrices not treated with exogenous DNase I did not develop color (data not shown), indicating that they do not contain significant amounts of endogenous nicks. (Bars = 10 μm.)

Examination of Other Cells. A variety of normal cells was examined by the *in situ* technique described here. The production of a deposit around the nuclear periphery was found to be a common occurrence in all normal cells tested. Of special interest was the fact that the normal human lymphocyte, a non-dividing cell, also exhibited a well-defined peripheral deposit of nuclear-exposed DNA (Fig. 4). Five other cancers examined all exhibited a decreased genome exposure at the nuclear rim, which was restored after reverse transformation (16).

Evidence for Existence of DNA Regions with Different DNase I Sensitivities. When the usual protocol for nick translation with DNase I was varied so as to treat the fixed cells for 20 min with the enzyme, cytoplasmic structures—presumably mitochondria—developed color (Fig. 5*C*) while the nuclear rim staining decreased markedly in intensity. Moreover, when application of the DNase I was done in a separate reaction before the incubation with polymerase and labeled nucleotide, it was possible to demonstrate that as digestion

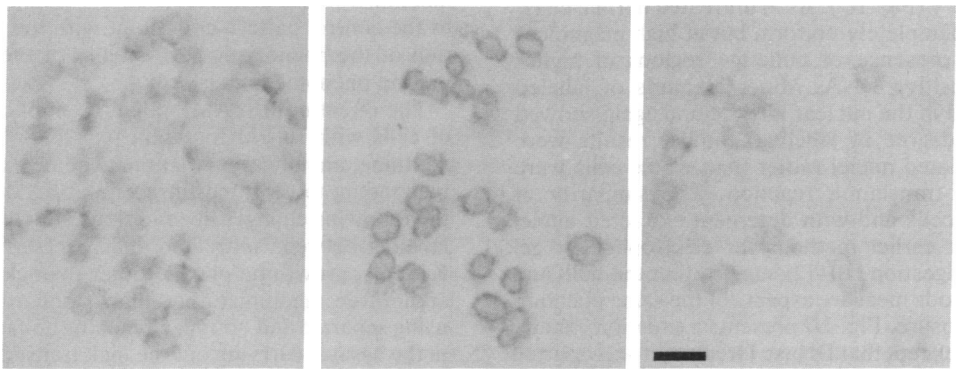

FIG. 2. Cytoskeletal involvement in genome exposure. Cells were grown for 3 days without additions (*Left*) or with 0.5 mM 8BrcAMP (*Center* and *Right*) and with 2.5 μM colcemid for 2.5 hr before fixation for assay (*Right*). The typical labeling pattern of reverse-transformed cells is eliminated in the presence of the microtubule-disrupting drug. (Bar = 20 μm.)

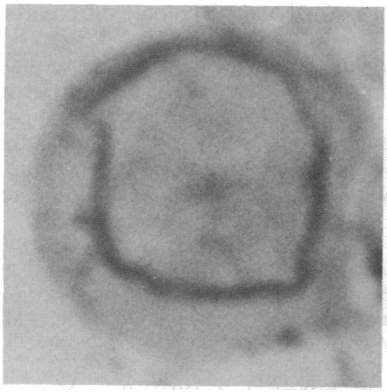

FIG. 4. *In situ* nick translation of a human lymphocyte from a cytospin preparation. Ring-labeling pattern typical of normal cell nuclei is seen. (Cell diameter = 10 μm.)

times were increased, a decrease in staining of the nuclear periphery and a concomitant increase in staining of an internal subset of chromatin occurred (Fig. 5*D*). The latter appeared to correspond in size and locale to centromeric chromatin (*Inset*). These results appear to indicate that the nuclear rim DNA is highly sensitive to digestion by the enzyme but that other classes of DNA with different degrees of sequestration exist within the nucleus. Thus prolonged treatment with DNase I completely removes the most sensitive DNA while gradually nicking more and more of the sequestered subsets.

Effects of Other Nucleases. Sensitivity of chromatin to digestion by DNase I is thought to involve alterations in nucleosomal structure (18) specifically detected by certain but not all endonucleases. Some experiments were done with restriction nucleases. Nicking of DNA of interphase CHO-K1 cells with low concentrations of restriction endonucleases *Eco*RI (Fig. 5*A*) or *Msp* I (Fig. 5*B*) followed by nick-translational incorporation of biotin-11-dUTP as a separate incubation step gave a pattern of labeling markedly different from that seen for DNase I. For all concentrations of these endonucleases tested (40–1500 units/ml), incorporation sites appeared distributed throughout the interphase nucleus.

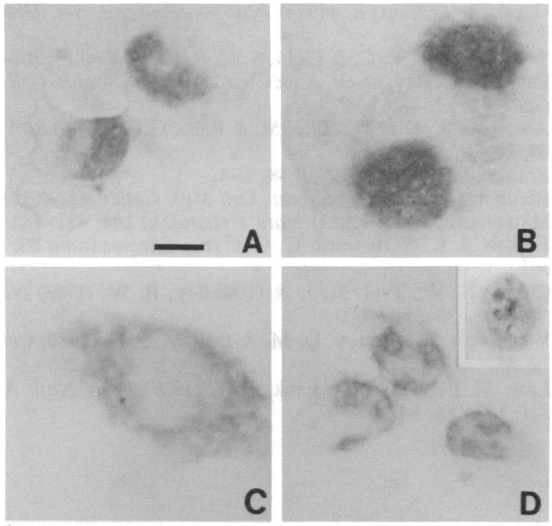

FIG. 5. Experimental variation of the labeling pattern. The patterns are described in the text. (*A*) *Eco*RI. (*B*) *Msp* I. (*C*) DNase I, 20 min. (*D*) Sixty-minute nicking incubation with DNase I followed by 1-min incorporation period. (*Inset*) Location of centromeric heterochromatin visualized by interphase C banding; Giemsa stain (17). (Bar = 10 μm.)

Thus, few preferentially labeled areas were seen with these sequence-specific (as opposed to conformation-specific DNase I) reagents. Moreover, these enzymes did not produce a labeling differential between control and 8BrcAMP-treated cells.

Reproducibility of the Reaction. Occasionally, experiments in these various studies failed to produce the expected differences between native CHO-K1 and its reverse transformed state. Thus, in 23 comparisons of the effect of cytoskeletal disruption by colcemid or cytochalasin B on the genome exposure action of 8BrcAMP, three failures to secure disappearance of the nuclear ring occurred (Fig. 2). Similarly, in 1 of 17 comparisons of the cAMP effect on nuclear matrix localization of exposed DNA (Fig. 3) no effect was seen. These occasional lapses are interpreted as an indication of the failure of current, still relatively crude, tissue culture procedures to yield maximal control over the genetic-biochemical cell parameters involved in these intimate regulatory phenomena.

DISCUSSION

These experiments show that in the CHO-K1 cell system normal cells have a typical nuclear rim of DNA that is highly sensitive to hydrolysis by DNase I (exposed DNA), the malignant cell has lost all or most of this exposed DNA, and the reverse-transformed cell has regained this subpopulation DNA with its characteristic high hydrolytic sensitivity and spatial distribution in the nucleus. These observations are undoubtedly related to those of others showing the DNase I sensitivity of active genes (19–21), but the magnitude of the transition displayed here in the difference between the cancer and normal cells suggests that the genome exposure reaction has further organizational consequences beyond new transcription of a small number of genes.

In an earlier paper (22) we have developed a theory of the regulation of the mammalian cell genome designed to explain the fact that mammalian cells, in contrast to simple cells like *E. coli*, exhibit differential patterns of gene activity in different tissues despite all cells of the body having the same chromosome and gene structure. A two-tiered level of regulation was proposed. In each differentiated tissue a unique spectrum of genes is considered to be exposed so as to be readily available to the action of modulating molecules like inducers, enhancers, and repressors. Genes specific for other tissues are pictured as sequestered—i.e., protected from such interactions through the action of nuclear protein structures. The genome exposure process was shown to involve a major fraction of the DNA, and an intact cytoskeleton was essential for the operation of the genome exposure mechanism.

The present experiments demonstrate that the lesion in genome exposure in the malignant CHO-K1 cell is largely located at the nuclear rim. With such malignancies the primary lesion would thus appear to lie in the genome exposure step of the regulatory mechanism rather than in the subsequent control of expression of the exposed genes. It is important to note that the regulatory lesion involves a decrease in genome exposure. Thus, it would appear that the genes that are erroneously sequestered in this form of cancer would be specific differentiation genes. Moreover, because cancer cells simultaneously lose specific differentiation characteristics and control of reproduction, these two sets of genes are probably genetically linked in differentiated cells.

We note that in all six different cancers tested we have found a similar relationship in which the cancerous state has lost a significant part of the exposed genome situated at the rim of the nucleus (16). One can conclude that this particular lesion is characteristic of at least a very important class of cancers. It becomes essential to extend these studies to a

large variety of malignancies. It is distinctly possible that these morphological findings will have diagnostic usefulness and that the principle of reverse transformation may find application in cancer therapy.

The existence of a definite protein complex associated with a nuclear ring of DNA has been well established (23, 24). The experiments of the present paper demonstrate the role of this important region of the nucleus in cancers that display the kind of behavior recorded here. It also follows that cAMP plays an essential role in the structure and functioning of this nuclear rim.

While it would appear that a nuclear rim with a particularly sensitive DNA fraction is a fairly generally characteristic in normal cells, our theory would favor interpretation that the specific DNA sequences present in this rim are different in each state of differentiation. Thus, the cellular "karyoskeleton" would appear to be part of the machinery at the convergence of the nuclear matrix, lamina, and membrane needed to localize the particular genes whose activity and modulation is required for each separate state of differentiation. These considerations imply that for each state of differentiation parts of particular chromosomes must take up specified positions in this critical region of the nuclear rim. Bennett (25) has demonstrated a peripheral interphase location of the parental chromosomes controlling phenotype in hybrid plant cells. The general theory that we have presented (22) and its further development here offer more detailed explanation of this phenomena and predictions for further experimentation. For example, one may well expect that along a particular differentiation pathway a successive set of genes will progressively undergo changes in their state of sequestration and exposure. The correspondence in the results between the technique described here and the results reported (10–12) with gel electrophoresis methods including the demonstrated need for cytoskeletal integrity in both assays as well as the large amount of DNA accessibility involved in the reverse transformation reaction supports the conclusion that the *in situ* assay described here measures the same DNA accessibility as measured by the gel analysis procedures. Hutchison and Weintraub originally described the *in situ* nick-translation technique in studies on mouse L cells and chicken and newt erythrocytes as model systems. Our results represent use of these techniques to visualize the changes in genome exposure in malignancy. In our hands normal and reverse-transformed fibroblasts and other normal differentiated cells gave fairly complete rings to the labeled nuclear sites rather than the partial rings described for L cells (9). It seems possible that the exposure mechanism of the immortal L cells have progressed away from the normal differentiation state but has not achieved the fully malignant condition characteristic of CHO-K1.

Several metabolic forms of DNA have been reported to be associated with the nuclear matrix including repetitive sequences (26), heterochromatin (27), transcriptionally active sequences (28), and replicons (29). Our laboratory has demonstrated differences in specific protein binding to repetitive sequence exhibited by the human cancer cell as opposed to normal cell lines (30). Further studies defining the specific DNA regions exposed at the nuclear rim under different conditions of normal and pathologic differentiation are now required.

The *in situ* methodology here described has several advantages over the previous gel electrophoretic methods used by us. It not only provides nuclear localization of the exposed genes but requires only a few thousand cells for each determination so that the methodology can now be applied to a wide variety of different normal and pathologic mammalian tissues.

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