Chapter 18

THE NUCLEOSKELETON: ACTIVE SITE OF TRANSCRIPTION OR ARTIFACT?

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I. CHROMATIN AGGREGATES UNDER ISOTONIC CONDITIONS

In an ideal world, a biochemist wishing to analyze structure-function relationships of DNA in the living cell would begin by selectively and progressively [illegible] the cell. First he might separate the nucleus from cytoplasm, isolate the chromatin, and finally purify the intact DNA free of all other constituents (Figure 1). This process might be achieved by treatments of increasing severity. (Note that in this field, terms such as "mild" and "severe" are almost always loaded terms!) However, under isotonic conditions this straightforward path as this *cannot* be traversed: the chromatin aggregates [illegible] and unworkable mess.[1,2] Even the superficially appealing approach of using a detergent such as sodium dodecyl sulfate to pass directly from cell to intact DNA is impossible — the released chromosomal DNA is so long and fragile it inevitably [illegible] by pipetting into small fragments.[3,4] (Biochemists nevertheless describe these [illegible] representing perhaps a one-hundredth part of the chromosome, as high-molecular-weight DNA!) Given the intractability of chromatin under physiological conditions, alternatives have been sought and used. These generally involve hyper- and hypotonic conditions or the presence of "stabilizing" cations. Such routes are unsatisfactory for several reasons. First, the "stabilizing" cations activate degradative nucleases. Template integrity and supercoiling are essential prerequisites for efficient replication, recombination, and transcription in simple templates,[5] so we might expect the same to be true of eukaryotic DNA. Second, unphysiological salt concentrations may introduce artifacts. That this is currently the central problem in this field is highlighted by studies on transcription. The traditional view of how transcription occurs is most dramatically illustrated by the photomicrographs of "genes in action" obtained by Miller and colleagues using hypotonic conditions.[6] These powerful images resembling Christmas trees are generally — but not always[7] — obtained with complexes containing polymerase I and are readily interpreted in terms of a mobile polymerase which processes along the DNA and is unattached to any larger structure. This model is extended to include complexes containing polymerase II and is now included in most standard textbooks.[8] Such a view is reinforced by the isolation of soluble polymerases that work in the absence of any larger elements. In sharp contrast, studies using hypertonic conditions suggest that nascent RNA is made as DNA passes through a polymerase fixed to some larger nuclear structure.[9] As a result, we have two paradoxical views of transcription: in the one, a skeletal substructure is the essential active site; in the other, it is not required and may not even exist. Similar differences in approach, results, and interpretation surround almost all aspects of higher-order structure in the nucleus and its relation to function.

II. VARIOUS SUBNUCLEAR STRUCTURES

A. Nuclei

Nuclei are usually isolated as the first step in any fractionation procedure in this field. Generally cells are hypotonically swollen and then broken by homogenization to release nuclei that can then be pelleted free of cytoplasmic contaminants. Divalent cations are added to prevent aggregation. It has recently been shown that this procedure — generally considered to be "mild" — extracts about a quarter of the protein and half the nascent RNA, roughly doubles nuclear volume,[10] and extensively nicks nuclear DNA.[11] Simply because this step has been considered a "mild" one, it is not so carefully standardized. Thus, different cell types are swollen — and so hypotonically extracted — for different periods and the released nuclei are exposed to endogenous nucleases, which vary in type and concentration from cell to cell, for different times.

B. Nuclear Matrices and Scaffolds

A wide range of subnuclear structures have then been extracted from such nuclei using

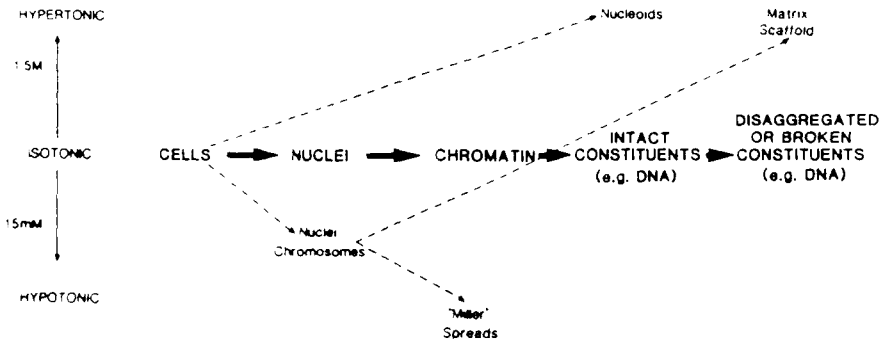

FIGURE 1. Various ways of isolating subnuclear structures. The solid arrows represent an ideal way of progressively deconstructing the cell into its components under isotonic conditions. In practice, the dotted lines, which depart from isotonic conditions, indicate the routes adopted.

nonionic detergents and high concentrations of salt (e.g., Triton X-100 and 2 *M* NaCl). They include nuclear pore complexes, envelopes, ghosts, matrices, lamins, scaffolds, and folded chromosomes. (For a review, see Reference 12.) Most share a basic set of proteins characteristic of pore complexes and lamins to which may be added other proteins, including part of the cytoskeleton. The pore complexes, envelopes, and lamins are derived mainly from the nuclear periphery and the matrix from the interior. To these various substructures, more or less degraded nucleic acid is attached. At one extreme are the pore complexes[13] and lamins[14] — essentially pure preparations of a few polypeptides; at the other are scaffolds which contain all the nuclear DNA, but this is broken (i.e., it is relaxed) and associated with some protein.[15] Nuclear matrices have an intermediate character, containing little RNA and DNA.[16] Recently — and confusingly — "matrices" have been isolated directly from cells using very different conditions from those used originally and as a result they contain most, and sometimes all, of the DNA and presumably many cytoskeletal elements.[17-20]

C. Nucleoids

An alternative approach is to lyse living cells directly in a nonionic detergent and a high concentration of salt.[21-23] Then structures are released which resemble nuclei depleted of many nuclear proteins. They contain all the nuclear RNA and DNA attached to a cage-like structure which contains the basic set of Triton- and salt-insoluble proteins found in the other subnuclear structures. However, they differ from these others in two very important respects: they have not been exposed to hypotonic conditions and their DNA is supercoiled and so largely intact.[21,23-25]

III. ARTIFACTS

A. The Problem

Currently, considerable attention is focused on these various subnuclear structures, not least because a role in nearly every nuclear function has been imputed to them: e.g., it is suggested that they pack the DNA into the nucleus and organize chromosome folding,[15] that they are the sites of replication,[18,26,27] transcription,[9] processing, and transport of nuclear RNA,[28-31] and that they are the target sites of regulatory molecules like steroid receptors,[32] calmodulin,[33] and viral T antigens.[34] While the gulf between such speculations and proof remains largely unbridged, the skeptic sees little need to ascribe any function to these *insoluble* structures. After all, DNA can be replicated and transcribed in vitro using much simpler and *soluble* systems. Are not these structures simply artifacts produced by exposure to extreme conditions (i.e., detergents and high salt concentrations)? For example, the

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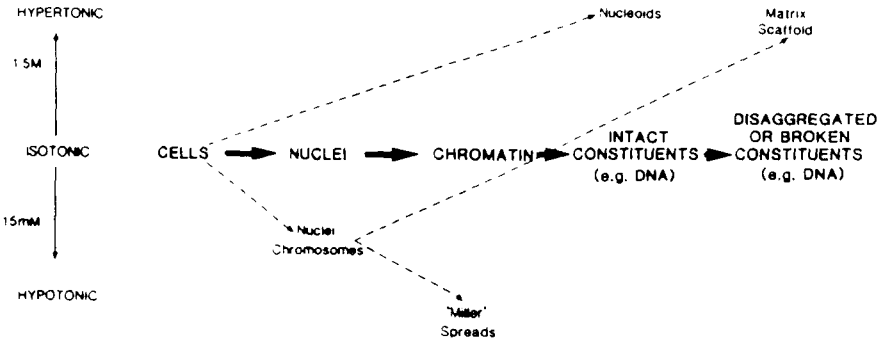

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striking images of chromosome scaffolds[15] could result from the nonspecific precipitation of protein onto a chromosome: far from the scaffold organizing the DNA, it is the chromosome that organizes the scaffold! Such artifactual association seems likely, especially as simple calculations show that RNA and DNA are present at extraordinarily high concentrations in the nucleus (about 100 mg/mℓ). Furthermore, single-stranded nucleic acids might be sticky and aggregate in high salt concentrations,[35] so one might expect RNA and nascent DNA to precipitate and condense onto any subnuclear structure. It is just by such an association that functional properties are inferred.

The problem, then, is to demonstrate whether or not any association seen is artifactual. The burden of proof clearly lies with workers in this field. One approach is to compare substructures isolated by different procedures; if an association in vitro is found consistently, then it is probably also found in vivo. However, this approach is constrained by the limited range of isolation conditions that can be used. As a result, skeptics remain unconvinced by the identity of structures isolated by replacing one counterion (e.g., Na^+) by another (e.g., dextran sulfate)[15] especially when other minor alterations in the sequence of operations or length of storage do profoundly alter the constitution of the resulting structure.[36,37]

A second approach, which has successfully correlated structures observed in vitro with those in vivo, is to raise antibodies to substructures — usually the simpler types like lamins — and to use them to probe the cellular distribution of the antigen by immunofluorescence. Such studies have confirmed that the lamin and envelope proteins are indeed nuclear or perinuclear and have highlighted how dynamic nuclear structure really is.[38-40] For example, while the lamina is reversibly depolymerized and dispersed during mitosis,[39] a 300-kilodalton matrix protein concentrates at the poles of the mitotic apparatus.[40] However, a nagging question remains — do not soluble antigens precipitate onto chromosomal structures during the isolation of any subnuclear structure?

B. The Essential Control — Demonstration of Specific Association

Biochemists in this field, just as in any other, must face up to the classic criticism of the physiologist or cell biologist — that on breaking open the cell they generate an artifact. This criticism can never be completely answered, but its force can be diminished if it can be shown that the particular artifacts with which one works are not just random aggregates of certain cellular components; more importantly, it must be shown that any associations between molecules seen in vitro are equivalent in both quantity and quality to those found in vivo. An artifactual association would seem unlikely if it could be shown (1) that *all* the molecules in the cell were associated and (2) that they were associated in a specific way.

IV. SPECIFIC ATTACHMENT TO SUBSTRUCTURES

A. Nascent RNA

When HeLa cells are incubated with [^{3}H]uridine for 1 min to label only nuclear RNA, >95% of the radioactivity initially present in the cells and insoluble in trichloroacetic acid subsequently cosediments with nucleoids.[9] A variety of experiments showed that this association was very tight, but is it specific?

A "cap" containing methylated bases is attached at the 5′ end of nascent RNA immediately that transcription begins, so the 5′ end of such transcripts can be labeled with [^{3}H] methionine. This label is also incorporated into proteins, DNA, and other methylated bases within RNA chains; 8.3% was demonstrated to be incorporated into caps during a pulse-label of 15 min. If nascent RNA is specifically attached at its 5′ end, caps should resist detachment by pancreatic ribonuclease. This is what was found: even when 75% of nascent RNA was detached, 8% of the label was recovered with cages in caps. (The difference between 8.3 and 8.0% can easily be attributed to differences in recovery of RNA chains of different

sizes.) It was concluded that the nascent RNA was specifically attached at its 5′ end to the cage.[9]

RNA molecules at later stages in their maturation are also found associated with the matrix,[28-31] but it remains to be shown in a similar way whether or not they are *specifically* associated.[41]

B. DNA

Attachments of DNA to a substructure have been inferred from the observation of supercoiling in nucleoid DNA.[21,42] Supercoils can only be maintained in linear DNA by looping it, presumably by attachment to a substructure. However, looping could occur by DNA-DNA or DNA-RNA-DNA interactions alone.[28,43] *All* the DNA can be isolated unbroken and attached only if cells are lysed directly in Triton and high concentrations of salt to yield nucleoids or "folded chromosomes" that have the greatest degree of contamination by cytoskeletal elements. Are such attachments specific? This can be answered by mapping the position of a sequence relative to the attachment point on the substructure — in this case the nucleoid cage — by detaching progressively more and more DNA from the cage with a nuclease. If attachments are specific, a sequence which lies close to the attachment site should resist detachment and so be enriched in the cage fraction; one lying further away should be depleted. On the other hand, if attachments are generated nonspecifically during isolation, they will vary from one nucleoid to another in the population so that any given sequence will neither be enriched nor depleted. It turns out that α-globin sequences attached to HeLa cages can be enriched approximately eight times, whereas the β- and γ-globin sequences in the same sample are depleted (i.e., the α-globin gene lies closer to an attachment site than the β- or γ-genes).[44] Active viral sequences in nucleoids from transformed cells are enriched even more (i.e., up to 18 times)[45] and the results of a typical experiment are illustrated in Figure 2.

This mapping technique has been extended to "matrix" DNA, with less convincing and sometimes conflicting results. Some groups have confirmed specificity of attachment; for example, they find integrated SV40 sequences,[17] the ovalbumin gene,[20] and some cellular repeats[46,47] enriched two to five times in the "matrix" fraction. Others, unable to demonstrate any convincing enrichment, argue that the attachments are artifactual.[37,48,49] Still others, by extracting nuclei with strong detergents, find different attachments to those found with matrices.[50] Clearly, the causes of these variations seen with "matrices" need to be examined and the more clear-cut enrichments obtained with nucleoids need to be confirmed. (Higher enrichments have been obtained at very high levels of detachment from matrices using "dot-blots".[37,51-53] However, technical problems with specificity and degree of hybridization of fragments of different sizes make interpretation of these results difficult.)

Even if genes can be mapped relative to attachment sites, these results could nevertheless be explained as follows. Nascent DNA or RNA — or the complexes generating them — could become sticky at high salt concentrations and so precipitate onto the substructure. As only a fraction of the genome is being replicated or transcribed at any time, only this fraction will precipitate, apparently specifically. If so, it would be unsurprising that nascent DNA and RNA are associated, or that sequences can be mapped relative to these association points. However, nascent DNA cannot be solely responsible for the attachments, since both loop size and the relative position of genes within a loop remain constant even when no DNA is replicating during mitosis or G1.[44,54] The criticism that transcribing complexes are the mediators of artifactual attachments is more difficult to eliminate, but some telling observations on the *specificity* of the attachments of nascent RNA make this possibility unlikely.

1. As described earlier, *all* the nascent RNA proves to be attached at its 5′ end.[9]
2. It is only RNA made in vivo in the nucleus that becomes attached: pulse-labeled RNA

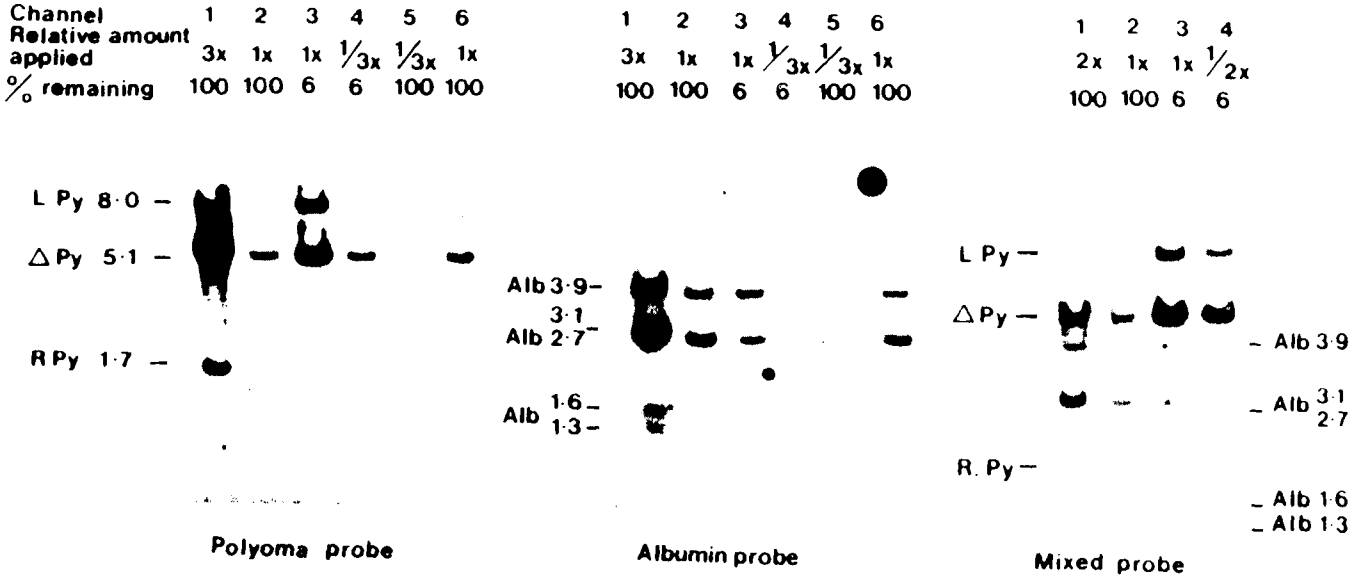

FIGURE 2. Detachment mapping of albumin and polyoma viral sequences in a rat cell transformed by polyoma virus (line 82). Some nucleoids from the transformed cell were partially digested with *Eco* RI, then cages — and the 6% of the total DNA that remained associated with them — sedimented free of detached DNA. Other nucleoids, incubated without *Eco* RI, yielded pellets containing all the DNA (i.e., 100%). DNA was purified from the two pellets and completely redigested using *Eco* RI. Various amounts of this DNA were resolved into discrete fragments by gel electrophoresis; these were transferred to a filter, hybridized with polyoma or albumin probes, and autoradiographs were prepared. The sizes of the 3 polyoma bands (LPy, ΔPy, and RPy, corresponding to the left-hand, internal, and right-hand fragments of the integrated virus) and the 5 albumin bands (2 are not clearly resolved under these conditions) are given in basepairs × 10^{-3}. The polyoma, but not the albumin, sequences are enriched in the nucleoid samples that retained 6% of the total DNA. This is highlighted by hybridizing a mixture of the two probes. (From Cook, P. R., Lang, J., Hayday, A., Lania, L., Fried, M., Chiswell, D. J., and Wyke, J. A., *EMBO J.*, 1, 447, 1982. With permission.)

does not associate with cages if first "chased" into the cytoplasm, nor does nascent RNA if synthesized in vitro, by *Escherichia coli* RNA polymerase, within the isolated nucleoid.[9]

3. Nascent transcripts of a nuclear virus, influenza virus, are all associated with cages, whereas those of a cytoplasmic rhabdovirus are not.[55]
4. The proximity of active viral genes in transformed cells to the cage cannot be an immediate consequence of transcription since they remain close to the cage when transcription stops during mitosis or heat shock (see later).

All these results concern nucleoids and should be extended to other structures.

V. ISOLATION OF CHROMATIN UNDER ISOTONIC CONDITIONS

A. Agarose Microbeads

Hitherto, we have been unable to selectively deconstruct cells and travel, under isotonic conditions, from cell to nucleus, from there to chromatin, and finally to the nuclear constituents (i.e., along the solid arrows in Figure 1) for two reasons. One is the susceptibility of DNA to shear and to endonucleolytic degradation, and the second is the tendency of chromatin to aggregate. Both problems can be solved by providing cells with an artificial and protective coat of agarose that is freely permeable to small molecules, proteins, and enzymes, but not to chromosomal DNA (Figure 3a). Subsequently, when relatively standard procedures for the selective deconstruction of the cells are followed, the agarose coat prevents aggregation and protects the fragile DNA (Figure 3b).[43]

Living cells can be encapsulated in agarose microbeads by homogenizing an aqueous phase containing cells in molten agarose with an immiscible phase of liquid paraffin; on cooling, suspended agarose droplets gel into microbeads.[56] The gelled beads can be removed from excess paraffin by centrifugation and then used directly or the cells can be cultured and grown within them. Bead diameter varies somewhat depending on the conditions used during homogenization: the standard procedure yields beads with about three fourths having diameters between 25 and 75 μm. Since less than one bead in 100 is more than 125 μm in diameter, these beads pass freely through the plastic tips used with automatic pipettes. The concentration of cells per bead can be varied widely; however, when beads are packed with cells (e.g., 10^8 per milliliter packed beads), many lie embedded on the surface and these tend to be detached on manipulation.

The pores in the beads offer no effective barrier to small molecules. For example, most cellular proteins and RNA diffuse freely through them.[10,43] Indeed, agarose beads are used routinely (e.g., as Biogel® A-150m from Bio-Rad®) as a filtration medium for fractionation of particles up to 150×10^6 daltons in chromatography columns. Particles of this size may also be electroeluted from the beads.[57] However, intact chromosomal DNA is too big to escape and remains trapped within the bead. As a result, if encapsulated HeLa cells are lysed using the conditions traditionally used to isolate nucleoids, the properties of the resulting encapsulated nucleoids are very similar to their unencapsulated counterparts.[43] Encapsulated cells have been treated with a wide range of different conditions to selectively extract various components from cells.[10] Two will be described in some detail.

B. Beads Containing Intact DNA

Nearly all protein and RNA can be stripped from DNA using ionic detergents (e.g., sodium or lithium dodecyl sulfate). When cells are so extracted, viscous DNA is released, forming a jelly which can only be manipulated after shearing and so breaking the DNA. If encapsulated cells are extracted with lithium dodecyl sulfate, the DNA remains trapped within the beads.[43] After washing, the resulting beads are found to contain all the cellular

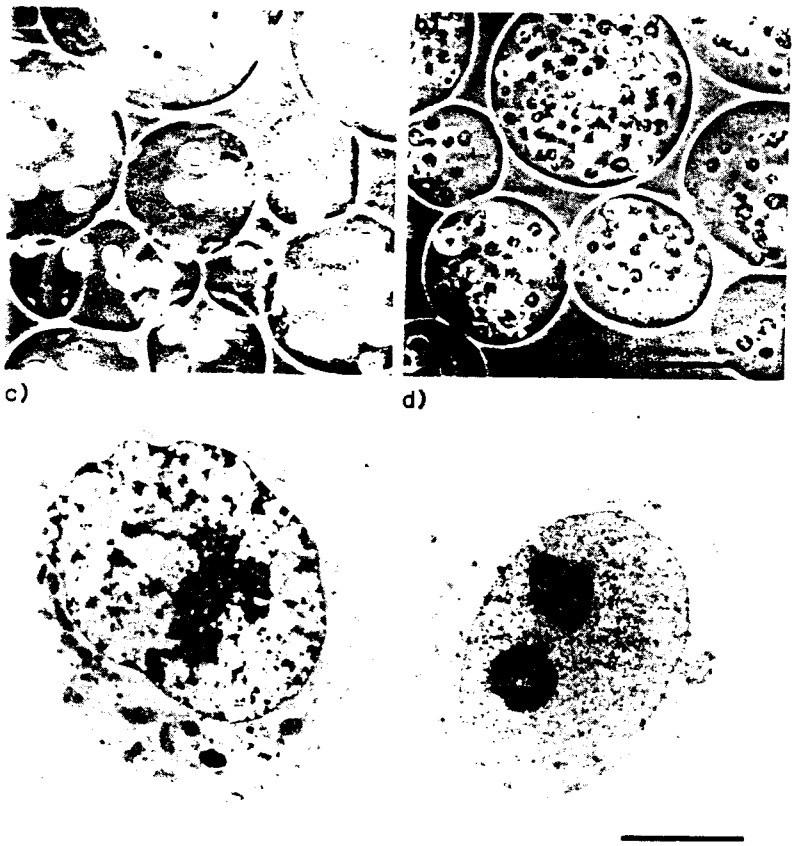

FIGURE 3. Phase contrast micrographs of agarose beads containing HeLa cells (a) or their derivatives lysed under isotonic conditions (b). Electron micrographs of the same samples are also shown in (c) and (d). The bars represent 100 (a,b),or 5 μm (c,d). (From Jackson, D. A. and Cook, P. R., A general method for preparing chromatin containing intact DNA, *EMBO J.*, 4, 913, 1985. With permission.)

DNA and less than 1% of the proteins. About 15% of the RNA remains and nearly all of this is nascent RNA which is probably complexed with the superhelical template (see below).

Remarkably, mitotic cells yield structures in which discrete chromosomes can be seen after staining with ethidium and which are stable for days on storage. Furthermore, the encapsulated DNA — whether from randomly growing or mitotic HeLa — is supercoiled because it binds ethidium in the manner characteristic of circular plasmid molecules. (At low concentrations, more dye binds to a negatively supercoiled DNA than to its relaxed counterpart. At high concentrations, where binding induces positive supercoiling, less dye binds to the intact molecule. This difference forms the basis of the widely used method for purifying supercoiled plasmid DNA free of relaxed plasmid or chromosomal DNA in cesium chloride density gradients.) This supercoiling is stable to electrophoresis, but relaxed by proteinase K. Presumably these supercoils are maintained in loops of DNA, and the kinetics of relaxation by γ-rays suggests that these loops are about 500 kb in size.

This survival of both supercoiling and chromosome morphology in lithium or sodium dodecyl sulfate was very surprising and begs a number of questions. For example, are the loops generated artifactually on lysis and do the loops have counterparts in vivo? It is easy to imagine how a long DNA molecule, ordered in a mitotic cell into a chromosome, might on deproteinization remain so entangled with itself that it retains some chromosomal mor-

phology. Fibers emanating from one tangle and then rejoining the same tangle later would be responsible for the apparent looped structure, with the tangles concentrated about rare, tightly bound protein molecules. According to this jaundiced view, the tangle in vitro would naturally reflect the structure in vivo, but only in the grossest sense. Of course, the same jaundiced eye sees the morphology of nucleoids, "matrices", and chromosome scaffolds in the same way.

However, might not these structures isolated in ionic detergents be closer to life than this? If loops are generated artifactually on lysis, we might expect the more tightly packed the DNA initially, the more tightly packed — and so smaller — the resulting loops. However, mitotic and interphase loops are similarly sized and do not progressively untangle and relax on storage or electrophoresis. Encapsulated chromosomes even retain their shape when subjected to an electric field in which the polarity is reversed every 30 sec. If these are not artifactual tangles, chromosomes — unlike scaffolds and matrices — must be maintained by forces resistant to strong ionic detergents.[43] Hydrogen bonds between nucleic acids involve just such forces, raising the possibility that chromosome morphology is maintained by single-stranded RNA or DNA bridging different parts of one duplex. An intriguing alternative is that a DNA molecule, *by itself*, can assemble into a chromosome. Perhaps specific interactions between identical sequences spaced along one duplex form the duplex into a series of loops. The close apposition of two identical duplexes to form a four-stranded structure in which two basepairs are themselves specifically paired (i.e., hydrogen bonded) is stereochemically possible[58] and stabilized by supercoiling.[24] The decision as to whether these structures isolated in strong ionic detergents are merely artifactual tangles or reflect rather precisely the ordering of DNA in vivo must await the results of experiments that address whether or not interactions at the base of these loops are *specific* or not.

C. Beads Containing Chromatin

If encapsulated cells are lysed under *isotonic* conditions using Triton X-100 and sufficient quantities of EDTA to chelate nearly all magnesium ions, structures resembling nuclei prepared by conventional procedures remain (Figure 3b).[10] Their DNA remains essentially intact, as judged by the ethidium-binding assay described earlier. Like isolated nuclei, their chromatin is less dense than that found in cells, and since they have been treated with Triton, they are surrounded by remnants of the nuclear membrane and cytoskeleton (Figure 3d). When analyzed by one-dimensional gel electrophoresis, the proteins of encapsulated nuclei are similar in amount and type to those of nuclei isolated by a conventional procedure, with the obvious addition of cytoskeletal elements. Their RNA contents are also very similar: both retain about 15% of the RNA labeled in 24 hr and essentially all that labeled in 2.5 min. How various procedures affect the attachment of radiolabeled RNA in encapsulated nuclei was examined by treating them and then removing any detached RNA by electrophoresis. Some of the RNA labeled in 24 hr is detached by hyper- or hypotonic treatment and nearly all is detached by ionic detergents. Perhaps surprisingly, the kind of hypotonic treatment (1.5 m*M* NaCl) that is widely used for the visualization of transcription complexes (i.e., in "Miller spreads") must destabilize the complex since about half the nascent RNA can subsequently be removed. Hypertonic treatment with 2 *M* NaCl detaches little nascent RNA and probably artifactually generates new attachments so that some become less easily extracted by lithium dodecyl sulfate. Even though sarkosyl extracts nearly all nuclear protein, about 30% of the nascent RNA resists extraction: presumably this is the fraction that is associated with the polymerase in the transcription complex.[59] Even after treatment with lithium dodecyl sulfate or proteinase K, some nascent RNA remains trapped in the bead, perhaps because it remains hydrogen bonded to the superhelical template.

Such encapsulated nuclei can be incubated on ice in buffers containing 1 m*M* EDTA for hours without further nicking. However, since such preparations might be used for functional

studies, their stability at 37°C in the presence of ATP and Mg^{2+} ions must be known. Incubation in 1 m*M* EDTA, 1 m*M* ATP, and 1 m*M* $MgCl_2$ for up to 30 min did not cause any nicking, but in 2 m*M* $MgCl_2$ the DNA became slowly nicked and at higher concentrations, more rapidly so. Since this assay for nicks is so sensitive — 1 nick per 200 kb giving the maximum effect — this means either that there is very little nucleolytic activity in these preparations, or that any nicking is efficiently reversed.

It should be stressed that the use of isotonic conditions does not necessarily guarantee the isolation of "native" nuclei or chromatin, only that the resulting preparation is likely to be less prone to artifacts than other preparations which use nonisotonic conditions. For example, the ionic balance within the nucleus must be disrupted, since the isolation procedure destroys the nuclear membrane. The availability of chromatin containing intact DNA, in an accessible yet stable and manipulable form, should prove useful for studies on higher-order structure in the nucleus and its relation to function. One such analysis is now described.

VI. TRANSCRIPTION OCCURS AT THE NUCLEOSKELETON

A. Models for Transcription

The model for transcription that is almost universally accepted involves a polymerase that processes along the template to generate a transcript that is attached to the polymerase and template at the nascent 3′ end.[8] This process takes place in the absence of any larger structure. Like many received ideas, it turns out that there is little *decisive* evidence supporting the model. Probably the best is provided by the photomicrographs of "genes in action" which resemble Christmas trees.[6] These beautiful images are usually obtained with complexes containing RNA polymerase I, but analogous complexes containing polymerase II can occasionally be seen.[7] Such "Miller spreads" are prepared by first isolating nuclei and then "gently" dispersing the chromatin in buffered distilled water.

Supporting evidence for this model is apparently provided by the many observations that soluble polymerases work in the absence of any larger structure. However, >95% of polymerase II pellets with broken nuclear fragments[60] and can be released only by prolonged incubation in Mg^{2+} ions or by sonication.[61] Furthermore, it is not widely appreciated that "semisoluble" polymerases initiate extraordinarily inefficiently: one of the most efficient systems, a crude "Manley extract", synthesizes correctly initiated transcripts at average rates of <10 nucleotides per hour[62] or 0.01% of the rate in vivo.[63]

An alternative view results from work on nucleoids isolated using hypertonic conditions.[9,55] All the RNA labeled with very short pulses is found associated with nucleoid cages and is attached at both the 3′ and 5′ ends (see earlier). If nascent RNA is so closely associated with the cage, then so too must be the genes from which it is transcribed: put in another way, DNA close to the cage should be richer than total DNA in transcribed sequences. This proposition was tested by preparing DNA by incubating nucleoids with *Eco* RI; then cages, and any associated DNA, were sedimented free of detached fragments to yield a pellet which retained 5% of the initial amount of cage-associated DNA. This cage-associated DNA was purified and the percentage forming a hybrid with an excess of total nucleoid RNA was determined: 23% was complementary to nucleoid RNA. Assuming that only one strand is transcribed, then about half this sample of cage-associated DNA contains transcribed sequences — a remarkable enrichment.

These experiments suggested that both ends of nascent transcripts were attached in some way and that transcribed sequences lay close to the cage. If so, transcripts might be generated as DNA passed through a fixed transcription complex which is associated with the cage. This model for transcription was tested in another way using a series of rat cells transformed by polyoma and avian sarcoma virus (ASV).[45] On transformation these viruses integrate randomly within the genome so that viral sequences might be expected to integrate initially

at random in the loops. However, if the cells express the transformed phenotype, one would predict that the integrated viral sequences, being transcribed, would lie close to the polymerase and so to the cage.

Sequences were mapped relative to their point of attachment using the procedure described earlier — the results obtained with the transformed lines are summarized in Table 1. (Of course, comparisons between different cells should be made at the same levels of detachment.) In no case is the concentration of untranscribed albumin sequences in the DNA which is closely associated with cages richer than that in the control. In every case the integrated viral sequences are enriched in the fraction of DNA that pellets with the cages.

One line — line 82 — was analyzed more extensively. In general, detaching more DNA from the nucleoids, whether with *Eco* RI or *Bam* HI, enriched all the viral sequences to a greater extent (Table 1) and the left-hand junction sequence, which contains both cellular flanking sequences and viral sequences, was enriched more than the internal, and purely viral sequence: both were enriched more than the right-hand junction sequence (Table 2). This suggests that the left-hand fragment is closest to an attachment site or is attached the strongest. Note that the left-hand, internal, and right-hand fragments contain 2, 1, and 0 viral enhancers, respectively.

Does the virus integrate selectively in sequences lying close to the cage or does it integrate randomly, inducing new attachments? Various viral sequences and contiguous cellular sequences have been cloned, therefore these possibilities were tested by seeing whether cellular sequences which flank the inserted virus lie close to the cage in the parental Rat-1 cells (Table 1). Cellular sequences homologous to all four such polyoma junction probes tested (i.e., 82J1, 53C1, 7TL, and 7TR) were readily detached from untransformed Rat-1 cages and cages prepared from ASV-transformants (i.e., the relative enrichments are <1.6) (Table 1). By contrast, in the polyoma transformants these cellular sequences were attached to the integrated viral DNA and so were clearly associated with the cage. The attachment of outlying cellular sequences induced by viral integration was highlighted as follows. The junction probe from the right side of the virus in 7axT (7TR) hybridizes with one major *Eco* RI fragment of 5 kb from parental Rat-1 cells. When the virus integrates, it does so into only one of the two homologous chromosomes, so that the junction probe now hybridizes to 2 fragments from the transformant 7axT — 1 of 5 kb from the unaffected chromosome and another of 5.1 kb, which contains viral sequences (Figure 4A). With total DNA, the 5 kb band is the more intense (Figure 4B, channels 1 and 2); however, when all but 4% of the DNA is detached from 7axT nucleoids, the band intensities are reversed (channels 3 and 4). The purely cellular 5 kb band is depleted while the viral 5.1-kb band is enriched. Clearly, the virus induces a novel attachment. A similar enrichment of the viral bands but depletion of the purely cellular band is obtained when the junction probe 82J1 is used with line 82 nucleoids.

Subclones of one of the ASV transformants (i.e., A11 V1T) enabled the strength of this correlation between gene activity and proximity to the cage to be tested more rigorously. Two subclones have lost the transformed phenotype and contain no detectable viral transcripts. When these "flat revertants" are treated with the antimetabolite, aza-cytidine, and recloned, transformed colonies containing viral transcripts emerge at a high frequency. As far as can be judged by restriction enzyme mapping, all cells in this series contain unchanged proviral sequences inserted in the same cellular sequence, but differ in whether or not the proviral sequence is expressed. Again, gene activity is found to correlate with proximity to the cage (Table 1).[20] This correlation even extends to hybrid cells formed by fusing two of the transformed lines with a normal cell. One hybrid is transformed, the other is not: in the one the ASV provirus lies close to the cage, in the other it does not (Table 1).

One trivial explanation of all these results is that nascent transcripts, which are presumably closely associated with their templates, prevent access of *Eco* RI to potential cutting sites in transcribing DNA. This possibility is unlikely since similar enrichments were seen when

Table 1
DETACHMENT MAPPING ALBUMIN, VIRAL, AND JUNCTION SEQUENCES IN VARIOUS CELL LINES

	Percentage DNA remaining (relative enrichment)			
Cell	Albumin (%)	Polyoma (%)	ASV (%)	Polyoma junctions (%)
Parent				
Rat-1	4 (1 ×)	100 (no bands)	100 (no bands)	9 (0.8 ×)[82J1]
				4 (1.6 ×)[53C1]
				9 (1.0 ×)[7TL]
				4 (1.2 ×)[7TR]
Polyoma-transformed				
82	6 (0.9 ×)	14 (1.7 ×)		
		6 (3.5 ×)		
		5 (6.9 ×)[a]		
		4 (4.6 ×)[b]		
		1 (6.7 ×)		
		0.8 (18.0 ×)		
53		6 (2.3 ×)		
7axT	4 (0.9 ×)	4 (4.0 ×)		
Tsa 3T3	5 (0.7 ×)	5 (3.0 ×)		
ASV-transformed				
A + 11	13 (0.6 ×)		13 (2.1 ×)	
A + 22	3 (0.8 ×)		3 (3.9 ×)	
A23	6 (0.5 ×)		6 (>3.0 ×)	6 (0.6 ×)[7TR]
B31	5 (0.6 ×)		5 (3.9 ×)	5 (0.5 ×)[7TL]
A11 V1T	14 (0.8 ×)		17 (2.1 ×)[c]	
	2 (1.0 ×)		14 (2.0 ×)	
			9 (3.0 ×)[d]	
			7 (3.0 ×)	
			5 (3.1 ×)[c]	
			5 (>9.0 ×)[b]	
			4 (7 ×)	
			2 (>9.0 ×)	
Flat revertants of A11 V1T				
13N			3 (1.4 ×)	
21N	10 (1 ×)		17 (1.0 ×)[b]	
			10 (0.9 ×)	
			10 (1.5 ×)	
			4 (0.8 ×)	
			4 (2 ×)	
Aza-cytidine selected retransformants of 21N				
21 aza-C trans 1			8 (5.6 ×)	
			7 (5.1 ×)	
21 aza-C trans 3			8 (7.7 ×)	
			7 (6.0 ×)	
			6 (4.5 ×)	
Nontransformed hybrid				
A23/B2E3			6 (0.6 ×)	
			2 (0.7 ×)	
Transformed hybrid				
B31/B2E3			9 (3.0 ×)	
			1.4 (7.8 ×)	

Table 1 (continued)

Note: Autoradiographs like those illustrated in Figure 2 were prepared for each cell line, using polyoma, ASV, albumin, or polyoma junction probes, and scanned using a microdensitometer, and peak heights were measured. The relative intensities of one of the strongest bands (and hence the relative enrichments) were determined by reference to similar bands obtained with varying weights of total DNA.[45,80]

[1] Nucleoids were obtained from a population containing 75% mitotic cells obtained by successive thymidine and colcemid blocks.
[2] *Bam* HI was used instead of *Eco* RI in both digestions.
[3] Nucleoids were incubated with ribonuclease to remove all but 4% or less of the RNA labeled in 15 min with [^{3}H]uridine (10 μCi/mℓ) prior to the first *Eco* RI digestion.
[4] Nucleoids were isolated from cells that had been subjected to 45°C for 10 min, a procedure that reduced incorporation of a pulse of [^{3}H]uridine into RNA by >95%.

Table 2
THE LEFT-HAND *Eco* RI FRAGMENTS OF THE INTEGRATED VIRUS IN LINE 82 LIE CLOSEST TO THE CAGE

Percentage DNA remaining	Relative enrichment of various fragments		
	Left	Internal	Right
14	1.7 ×	1.7 ×	1.6 ×
14	2.0 ×	1.4 ×	1.0 ×
6	3.5 ×	3.0 ×	2.2 ×
1	6.7 ×	5.5 ×	—
0.8	18.0 ×	13.7 ×	10.0 ×

Note: Band intensities in autoradiographs prepared like those in Figure 2 were measured and the relative enrichments were determined.

From Cook, P. R., Lang, J., Hayday, A., Lania, L., Fried, M., Chiswell, D. J., and Wyke, J. A., *EMBO J.*, 1, 447, 1982. With permission.

1. *Bam* HI replaced *Eco* RI in both digestions.
2. Nascent RNA was detached prior to *Eco* RI digestion.
3. Transcription was suppressed by heat shock or during mitosis (Table 1).

B. Testing the Models

The essential difference between these two models for transcription is the participation of a larger nuclear substructure at the active site of the transcription complex (Figure 5). In theory they can be distinguished using encapsulated chromatin prepared under isotonic conditions, by fragmenting the chromatin with an endonuclease and finally removing any unattached chromatin by electrophoresis. If the polymerase is unattached to any larger structure, then all three constituents of the transcription complex, the nascent transcript, active RNA polymerase, and the transcribed gene, should all escape from the bead on electrophoresis (Figure 5A). Indeed, we might expect even low levels of digestion to lead to the preferential loss of all three, since active genes are preferentially cut by endonucleases.[64] On the other hand, if the polymerase is attached, all three should remain associated with the larger structure since it is too big to escape from the bead (Figure 5B). It turns out

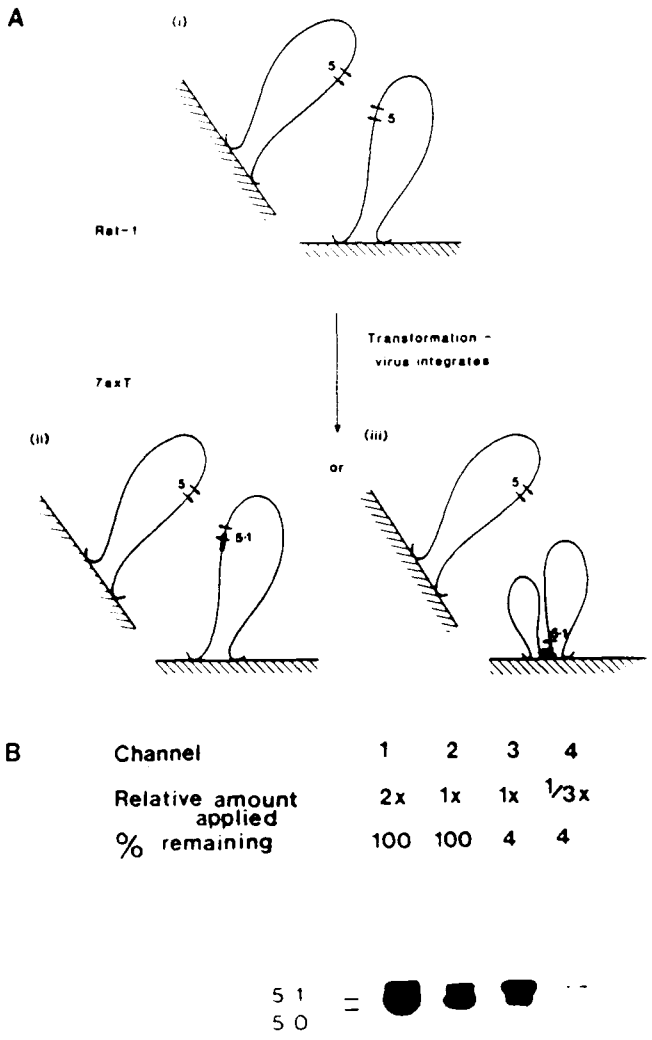

FIGURE 4. Detachment mapping of a junction sequence (7TR) in 7axT. (A) Two loops on each of two homologous chromosomes of uninfected Rat-1 cells are shown (i) attached to the nuclear skeleton (hatched area). On transformation by ASV, the virus integrates into one of the loops (solid rectangle) to yield line 7axT. This line might have unaltered attachments (ii) or contain a novel attachment (iii). The junction sequence (7TR) of 5.1 kb from 7axT hybridizes to the 2 homologous 5-kb *Eco* RI restriction fragments in Rat-1 and to fragments of 5 and 5.1 kb in 7axT (from the unaffected and affected chromosomes, respectively). (B) Various amounts of total DNA or DNA which resisted detachment by *Eco* RI (4% remaining associated with cages) were applied to the gel. Autoradiographs were prepared using the junction probe 7TR. In the cage-associated sample (channels 3 and 4), the 5.1- and 5-kb bands are enriched 2.4 × and 0.6 ×, respectively. (From Cook, P. R., Lang, J., Hayday, A., Lania, L., Fried, M., Chiswell, D. J., and Wyke, J. A., *EMBO J.*, 1, 447, 1982. With permission.)

that all three remain trapped within the bead consistent with Figure 5B.[57] A typical experiment demonstrating that little, if any, active RNA polymerase II can escape is now described.

Cellular DNA was uniformly labeled with ^{3}H, the cells were encapsulated, lysed under isotonic conditions, and RNA polymerase activity was assayed by incorporation of [^{32}P]UTP into acid-insoluble material. The resulting encapsulated nuclei contain active RNA polymerase II, which is halted by lysis during elongation on the endogenous template. This

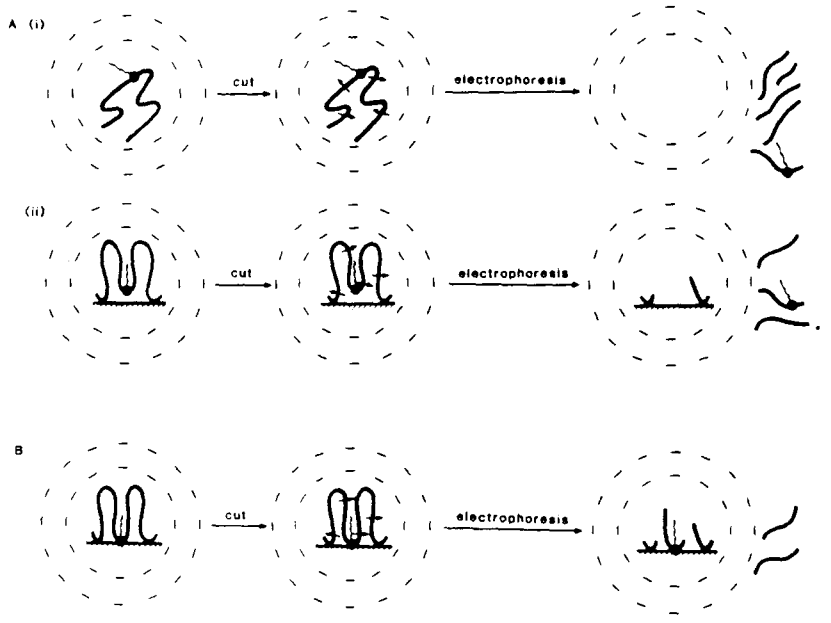

FIGURE 5. Two models for transcription. (A) RNA polymerase (●) processes along the DNA (—) synthesizing a nascent transcript (wavy line). Two alternatives are depicted — in (i) the nuclear substructure has no role, while in (ii) it organizes the chromatin into looped domains. (B) The same looped domains are present in (B), but now transcripts are generated as DNA moves past a polymerase which is also associated with the nuclear skeleton (hatched area). A variant of (B) is that the 5′ end of the nascent transcript is also attached in some way.[9] After cutting DNA with an endonuclease (arrow) and electrophoresis, nascent RNA, polymerase, and active genes should be retained within the beads (broken circles) in (B), but not (A).

polymerase does not reinitiate, but elongates the existing nascent RNA chains under these conditions. *Eco* RI digestion of the encapsulated nuclei reduced the total amount of RNA made in vitro to 85% of that of the control, presumably because the template was truncated or its conformation otherwise changed. Subsequent removal of 70% of the chromatin by electrophoresis reduced the activity no further. Therefore the polymerase resists removal, unlike the majority of the chromatin. Although the chromatin fiber has been cut into pieces small enough to escape, perhaps the nascent RNA and associated protein made the transcription complex too bulky. Therefore, beads were incubated with sufficient RNase A to detach >95% nascent RNA and hence RNP. With or without electrophoresis this reduced the polymerase activity by about 15%. Treatment with both RNase and *Eco* RI, followed by electrophoresis, removed >95% nascent RNA and 73% of the DNA. In contrast, 70% of the polymerase activity remained and this reduction by 30% could be explained by the additive effects of *Eco* RI and RNase treatments (i.e., 15% plus 15%). Clearly, little if any active polymerase escapes on removal of 73% of the chromatin and more than 95% of the degraded RNA and associated ribonucleoprotein. This suggested that the transcribing complex must remain very large, too large to pass through the pores in the bead.

These results naturally beg the question: to what is the complex attached? However, these studies did not answer this question; they only suggested that the structure resists electroelution and so is probably very large. However, it seems likely that the nuclear cage and matrix are intimately related to it. The term nucleoskeleton was used to describe the structure found under isotonic conditions to distinguish it from the others isolated in 2 *M* NaCl, and it was suggested that the nucleoskeleton was the structure with which the transcription

complex is associated. Obviously, further work is required to define the nature of the nucleoskeleton and its proposed interactions with the transcription complex.

Transcription complexes seen in spreads after exposure to hypotonic media are not attached to a larger structure, whereas their counterparts isolated in 2 *M* NaCl are. These apparently paradoxical results can be reconciled if it is accepted that *both* are artifacts. In 2 *M* NaCl, nucleosome structure and polymerase activity are destroyed, while attachments of active genes and nascent transcripts to the nucleoskeleton are retained. Indeed, new attachments may be generated.[10,50] On the other hand, in hypotonic media, nucleosome structure and polymerase activity are retained, but the transcription complex is disrupted and some transcripts are detached from the nucleoskeleton. Since hypotonic treatment removes one quarter of the protein of encapsulated nuclei[10] and since no skeletal structure can be seen in "Miller" spreads, it may even be destroyed. Of course, it may turn out that polymerase II transcription units are attached, while the polymerase I transcription units usually seen in spreads are not. In this context it is worth noting that both polymerase I and III (unlike polymerase II) are found in the soluble fraction when nuclei are prepared. This suggests that if these two enzymes are attached, they and polymerase II must be attached differently.

VII. TRANSCRIPTION OF LOOPS OR CIRCLES

Transcription of loops or circles poses a number of topological problems[9] which apply equally to models involving mobile polymerases or mobile templates. (One must necessarily move relative to the other.) Imagine the polymerase of *Escherichia coli* transcribing a supercoiled plasmid DNA molecule (e.g., πVX), of about 1 kb in length, by processing along 1 strand of the double helix. Such a circular template would have a radius of about 9 nm if condensed 6-fold by supercoiling. If the transcript encodes a polypeptide, the enzyme (radius about 7.5 nm), plus transcript, attached ribosomes (each with a radius of about 15 nm) and nascent protein must *all* pass once through the center of the circular template on transcription of every turn of the helix. Even if they managed to do so, the resulting transcript would be intertwined around the template once for every helical turn transcribed, and could only be separated completely from the template by rotating one end about the template axis (once for every turn transcribed). As this whole process seems implausible, these problems must be sidestepped. The intertwining, but not threading, problem would be solved by attaching both ends of the nascent transcript to the polymerase: then the 3′ and 5′ ends necessarily rotate about the helix axis the same number of times. Both problems would not arise if there was no net rotation of polymerase and template about each other. This could be achieved by a discontinuous elongation process in which the polymerase transcribed one turn of the helix, then paused, and rotated once back around the helix axis before repeating the process. Alternatively, the polymerase might transcribe a separated or "melted" template strand which did not intertwine locally around the other. If no covalent bonds were broken, then the melted turns must necessarily be stored elsewhere in the template. This could be achieved by altering its supercoiling, but it is difficult to imagine how sufficient supercoils could be stored during the synthesis of long transcripts. If covalent bonds in the template were cut and resealed, then melted turns could be removed and reintroduced (e.g., by a nicking-closing activity) during transcription.[9,65] Another possibility is that covalent bonds in the transcript are cut and resealed, with the template passing through the cut. In eukaryotes such a cutting and resealing occurs during splicing.

These formal possibilities are listed here to emphasize the constraints that apply to *any* model of transcription whether applied to prokaryotes or eukaryotes. It seems to us that the most likely solution involves

1. No net rotation of polymerase and transcript about the helical axis simply because they are too bulky

2. A consequent obligatory untwisting of the template before it reaches the polymerization site
3. Retwisting afterwards

VIII. AN ATTACHMENT HYPOTHESIS FOR TRANSCRIPTION

We envisage the nucleoskeleton as one part of the active site of the transcription complex, organizing the template — the DNA — in three-dimensional space into close proximity to the polymerization site. Transcription proceeds by passage of the DNA through the complex to yield attached transcripts. If the polymerase is tethered to the nucleoskeleton, then genes closely associated with this skeleton will be transcribed in preference to those that are remote from it (Figure 6). Then it becomes easy to imagine how selective attachment of genes to the nucleoskeleton might underlie selective gene activity during development or oncogenesis.[9,10,66]

The attachments and detachments of the integrated viral genes described earlier are entirely consistent with this hypothesis, as is the close association of the expressed ovalbumin gene — but not its unexpressed counterpart — with the "matrix".[20] An infecting virus wishing to subvert the cellular transcription machinery would have to attach to the nucleoskeleton, and this is indeed true of influenza virus.[55] The hypothesis also allows a reinterpretation of some old data on the structure of DNA in nucleoids derived from various cells of the avian erythrocyte lineage.[22] Development from the immature and transcriptionally active erythroblast through the reticulocyte to the mature but inactive erythrocyte is marked by the disappearance of the nucleoid cage and a progressive loss of supercoiling (perhaps detachment) of the DNA. When the inert erythrocyte nucleus is reactivated by fusing the erythrocyte with an active cell, elements of the nuclear matrix reappear.[67] The inactivation and then reactivation of transcriptional activity correlates with the disappearance and then reappearance of the cage or matrix; it also correlates with the progressive detachment and (presumably) reattachment of the DNA.

What might trigger specific attachments of target sequences during development is completely obscure and could involve any one of three formal possibilities. First, their chemical constitution could be altered, e.g., by rearrangements like those involved in the activation of immunoglobulin genes[68] or by modifying (e.g., hypomethylating) their DNA.[69] Second, their affinity for the nucleoskeleton might be altered by changing their conformation, perhaps by coiling or supercoiling them in a different sense or degree.[70-72] The third is the traditional one which involves the selective binding of specific attachment proteins.[7] The availability of chromatin prepared under isotonic conditions should allow us to isolate attachment sequences and their binding sites, so enabling us to distinguish between these possibilities.

Switching genes on by attachment can be reconciled with old ideas on the importance of a critical cell division prior to restriction in developmental capacity. If specific sequences become permanently attached as they are replicated at the nucleoskeleton, the order in which adjacent genes pass through the replication complex might determine their sequence of expression during development. Interestingly, the α- and β-globin gene clusters are probably each in one loop in HeLa cells,[44] and genes in both clusters are arranged along the chromosome in order of their expression during ontology.[74]

What sequences might be involved in bringing the gene to be transcribed close to the polymerase complex? They would be expected to increase the rate of transcription dramatically and to be *cis* acting, and these are just the properties of "enhancer" sequences.[75,76] Current thinking views these as "entry" sites for a mobile polymerase: instead, they might be entry sites for the mobile template, bringing it close to a tethered polymerase.

Polymerase attachment has a number of other interesting consequences. For example,

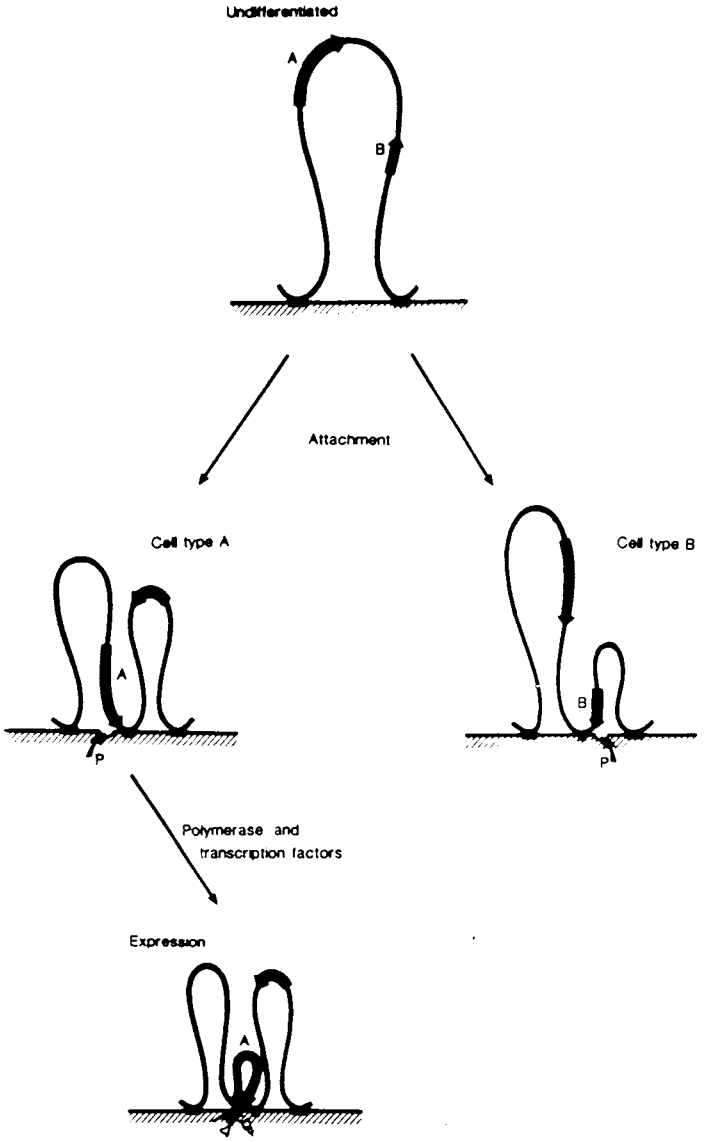

FIGURE 6. An attachment model for differentiation. In the undifferentiated cell, nuclear DNA is specifically attached (▬) to the nucleoskeleton (hatched area) forming loops. During differentiation, novel functional attachments (▲) specific to a cell type are generated, bringing specific genes (A or B) close to a polymerization site at the nucleoskeleton (P). In the presence of polymerase and appropriate transcription factors, the attached gene is transcribed as it moves past the polymerase, generating an attached transcript.

only certain sequences — perhaps only those within one loop of DNA — will be accessible to any one enzyme or cluster of enzymes. Put in another way, a polymerase will be dedicated to transcribing a few, and perhaps only one, transcription units. Furthermore, there will be structural constraints on attaching two adjacent promoters. Hence, when they are very close together it is possible that stable DNA-nucleoskeleton interactions allow only one to function at a time — precisely this type of effect has been reported.[77] Increasing the distance between

the two promoters, so that they can both loop back to the nucleoskeleton, might enable both to function.

According to this hypothesis — obviously highly speculative — genes within nuclei are organized in a quasi-crystalline manner, and *where* a gene is in three-dimensional space — its proximity to active sites on the nucleoskeleton — critically determines its function. Different tissues within an organism would be characterized by different arrays of attachments, but within a tissue the three-dimensional structure of DNA would be very similar from cell to cell. It is easy to imagine (in a general sense) how gross structural changes of the type described might be duplicated when the chromosome was duplicated and be sufficiently stable to persist through mitosis, so that a particular differentiated state might be stably inherited by the mitotic descendents of a particular cell.[70,71] In addition, such gross structural changes might be expected to induce more subtle changes in chromatin conformation, e.g., making active genes sensitive or hypersensitive to nucleases.[64]

A hypothesis in which attachment is a necessary precondition for transcription is readily extended to include replication, the repair of damaged DNA,[27,78] and recombination. Such a general hypothesis might appeal to a mariner, who knows that ropes of any length must be tied down at all times, otherwise they become tangled; and if that mariner wished to work on a piece of rigging, he would first bring that piece down to the deck and secure it firmly. The same may be true of the long nucleic acid polymers in the nucleus.

IX. CONCLUSIONS

The field of higher-order structure in the nucleus has been bedeviled with the problem of whether or not a particular structure is an artifact. To a large extent this has been due to our inability hitherto to isolate nuclei and chromatin under physiological conditions, while preserving the integrity of the DNA. Now, however, it is possible to isolate chromatin under isotonic — if not physiological — conditions, so that we can be more confident that some of the controversies will soon be resolved.

ACKNOWLEDGMENTS

Our work is supported by the Cancer Research Campaign. We thank the editor of *EMBO J.* for permission to reprint figures 2, 3, and 4B.

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