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Review

The nucleoskeleton and the topology of transcription

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Transcription is conventionally believed to occur by passage of a mobile polymerase along a fixed template. Evidence for this model is derived almost entirely from material prepared using hypotonic salt concentrations. Studies on subnuclear structures isolated using hypertonic conditions, and more recently using conditions closer to the physiological, suggest an alternative. Transcription occurs as the template moves past a polymerase attached to a nucleoskeleton; this skeleton is the active site of transcription. Evidence for the two models is summarised. Much of it is consistent with the polymerase being attached and not freely diffusible. Some consequences of such a model are discussed.

The accepted model for transcription contains three essential participants – the template, polymerase and nascent RNA [1, 2]. Transcription is initiated by a diffusible polymerase binding to a promoter; the polymerase then processes along the DNA (Fig. 1 A). As knowledge has increased, other occasional participants have been added (e.g. transcription factors, topoisomerases) but not any other central players. Evidence for this model comes almost entirely from studies using hypotonic salt concentrations, largely because chromatin aggregates in physiological concentrations and so becomes difficult to use. In contrast, studies on preparations isolated using hyper- or iso-tonic salt concentrations suggest that active RNA polymerase is associated with a nucleoskeleton and is not freely diffusible. This nucleoskeleton is seen as the active site of RNA synthesis and transcription occurs by movement of DNA past the attached polymerase (Fig. 1 B).

Ultimately models will be distinguished by reconstructing efficient transcription *in vitro* from purified constituents. Proof of the model involving an attached polymerase will require measurement of its association constant for a skeleton. In the meantime, the two models can be distinguished operationally by determining whether the polymerase is freely diffusible or attached to a larger structure. Much evidence for the 'text-book' model is consistent with the alternative; this alternative has a number of important consequences and these are examined. Discussion centres on RNA polymerase II, the enzyme that transcribes most eukaryotic genes, but applies equally to other polymerases.

ARTEFACTS

Structure within isolated nuclei cannot be discussed sensibly without some consideration of problems caused by artefacts. They arise because nuclei and chromatin aggregate in physiological salt concentrations [3]; therefore unphysiological conditions are almost invariably used. Controversy centres on whether structures seen *in vitro* are any more than isolation artefacts. RNA, DNA and protein, each concentrated in the nucleus at about 0.1 g/ml, might be expected to aggregate as soon as ion concentrations are altered. (For reviews of the controversy, see [4–6].) Nuclei are usually isolated by lysing cells in about 1/10 the physiological salt concentration [7]. Intuition suggests that such low concentrations are 'mild', but they destroy the 30-nm chromatin fibre, decondense heterochromatin, extract a quarter of nuclear protein (including active polymerases [8, 9]) and aggregate ribonucleoprotein particles [10]. The 'stabilizing' cations that are usually added also activate nucleases, so supercoiling is lost and polymerases can initiate aberrantly at resulting nicks.

These, then, are just the initial conditions that are traditionally used. Subsequently these hypotonically extracted nuclei are themselves re-extracted to prepare various subnuclear structures. For example, 'matrices' are prepared by extraction in 2 M NaCl (for a review see [11]) and 'scaffolds' by extraction with lithium diiodosalicylate following a mandatory heat-shock [12]. What relationship such structures bear to those *in vivo* is open to argument. Ultimately the criticism that artefacts have been generated are best countered using physiological conditions, so discussion will concentrate on the few studies which use them.

EVIDENCE FOR THE 'TEXT-BOOK' MODEL

Despite almost complete acceptance of the 'text-book' model, there seem to be only two kinds of evidence to support it.

'Miller' spreads

'Miller' spreads apparently provide the best direct evidence [13]. They are prepared by dropping nuclei isolated using conditions described above in a solution that is little more than distilled water (sometimes containing the detergent 'Joy'). Removing counterions charges chromatin, which expands and bursts the nucleus; individual chromatin fibres and

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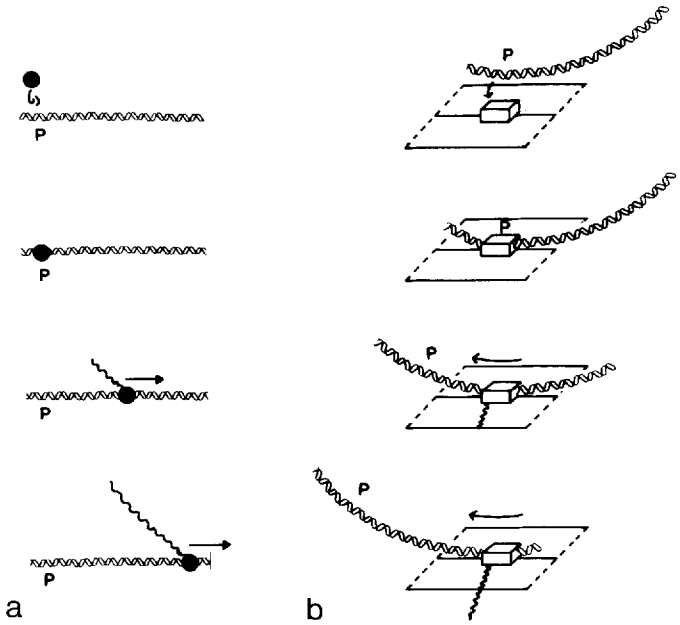

Fig. 1. *Two models for transcription.* Four different stages in the process are shown (top to bottom): formation of the polymerase–template complex, initiation of RNA synthesis at P and elongation of RNA. (A) The 'text-book' model. The polymerase (circle) moves along a fixed template to generate the transcript (wavy line). (B) The attachment model. The template moves past a polymerase (rectangular box) attached to a nucleoskeleton (grid)

'text-book' transcription complexes can then be seen at the edge of the spread chromatin. The most striking of these resemble Christmas trees and contain highly transcribed genes like the silk fibroin gene (Fig. 2a) or ribosomal DNA, a polymerase I unit. No skeleton can be seen.

A priori, it would seem dangerous to draw general conclusions about structures *in vivo* using such a disruptive procedure and based on visualization of a minority of transcription complexes.

Soluble polymerases

Modern biochemistry has proved very successful at dissecting details of transcription [14–16] and this very success lends support to the generally accepted model; if soluble polymerases work, then the model must be correct.

It is the general experience of those that purify RNA polymerase II that most enzyme is insoluble [17, 18]. A large pool of soluble enzyme is found in frog's eggs [19] but this is inactive, awaiting activation later in development. It is rarely appreciated how inefficient such soluble polymerases are. Rates of transcription *in vitro* cannot be expressed relative to rates *in vivo* as the latter are not known, since rates of RNA turnover cannot be assessed accurately. Additionally, extremely low absolute synthetic rates can be measured because ^{32}P-labelled precursors of high specific activities are available. Overall efficiencies are then easily overlooked. Pure polymerases transcribe 'activated' templates at > 50 nucleotides/s, initiating incorrectly at nicks. In contrast, they are almost completely inactive on intact chromatin. Systems that do initiate correctly are impure, involving long preincubations in crude extracts; one of the most efficient mammalian systems, a 'Manley' extract, polymerises correctly initiated transcripts at < 10 nucleotides/h, or perhaps 0.01% of the rate *in vivo* [20]. In a yeast system, $< 10^{-3}$ transcripts are accurately initiated per added template molecule during a typical incubation [21]. Chromatin templates are transcribed even less efficiently [22, 23] but appropriate preincubations improve rates slightly [24]. Quite possibly, some templates assemble into larger structures during preincubations and prime what little specific transcription there is.

Until a soluble system is developed that initiates correctly at rates approaching those found *in vivo*, this kind of evidence cannot provide definitive proof for a skeleton-free model. Indeed, observed activities seem to be partial reactions, lesser and non-specific activities resulting from disruption of some larger and more active complex. It is also as well to remember that the largest stimulatory effects seen to date *in vitro* are small when compared to effects that occur during development. Thus, sequences like enhancers and upstream activating sequences stimulate transcription *in vitro* by factors of a few hundred or less; they have similar effects in 'transient expression' assays. These are to be compared with the 10^8-fold differences in rate of transcription of the growth hormone gene that probably occur during development [25].

LOOPS AND SUPERCOILS IN NUCLEAR DNA

The organization of the chromatin fibre into loops or domains is an essential part of the alternative model. It is also a recurrent motif in many chromosome models. Again, despite wide acceptance, evidence for looping is inconclusive and controversial, derived almost entirely from studies using unphysiological conditions.

Early evidence

The best evidence remains the observation of meiotic lampbrush chromosomes in living amphibian cells, where lateral loops of native chromatin are specifically attached to a central core [26]. However, transcription here is very odd as globin genes [27] and both strands of some repeated sequences are transcribed promiscuously [28, 29].

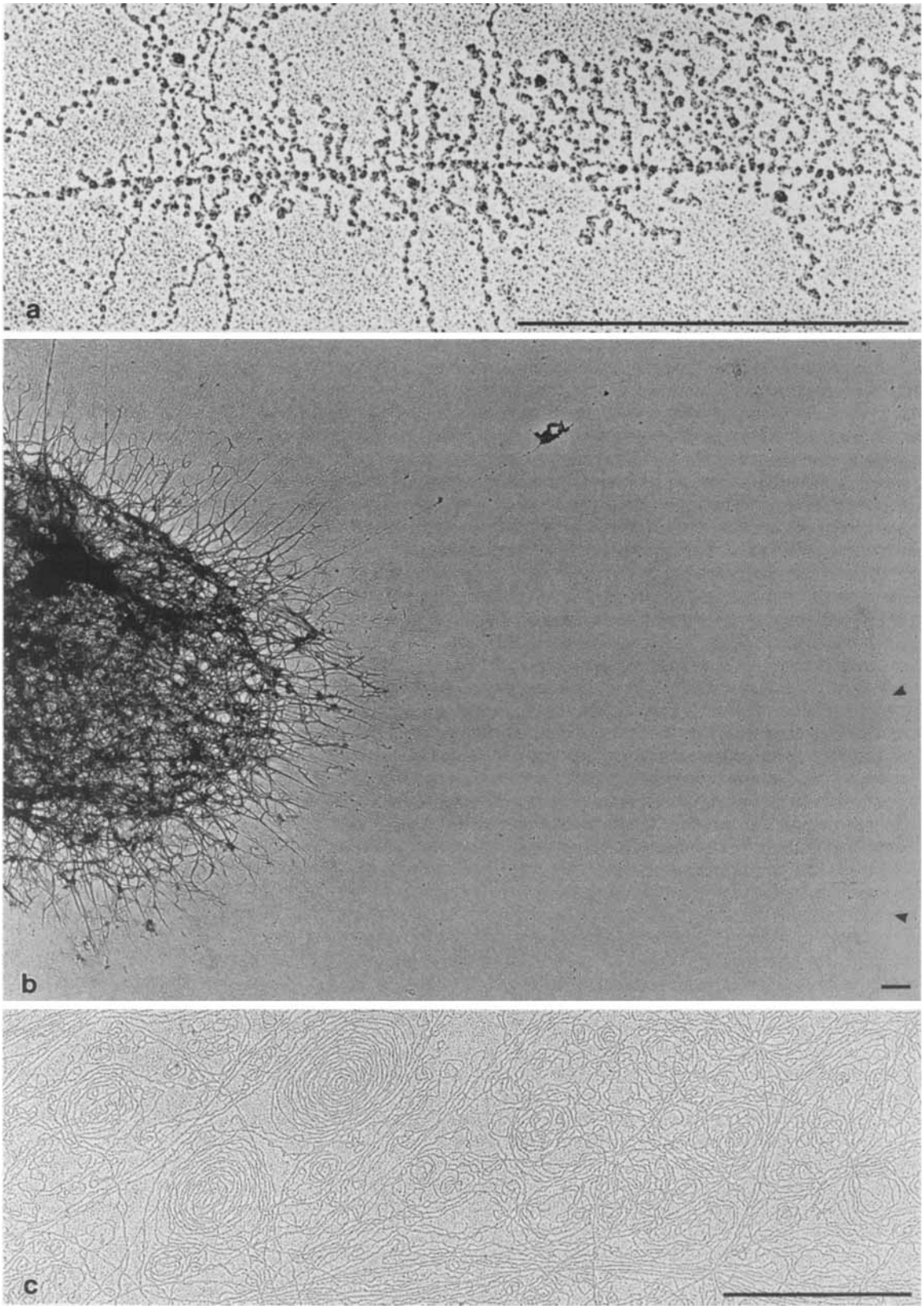

Fig. 2. *Electron microscopy of nuclei prepared under hypotonic (a) or hypertonic (b, c) conditions gives very different views of how transcription might occur.* (a) Part of a 'Miller' spread showing the silk fibroin transcription unit. Chromatin is dispersed by diluting counterions in buffered distilled water. A typical 'Christmas tree' structure is visible, but no 'nucleoskeleton'. From McKnight et al. [236] with permission. (b) Low-power view of a 'nucleoid' prepared by lysing cells in 2 M NaCl and then spreading DNA using Kleinschmidt's procedure. A tangled network of superhelical fibres extend from the prominent central skeleton (left) to the edge of the field (arrowheads). See [47]. (c) High-power view of the edge of a spread like that in (b). No nascent RNA can be seen amongst the DNA, all remains associated with the central skeleton in (b). From McCready et al. [47] with permission. Bars are 1 μm

Loops are more generally seen in fixed mitotic or polytene chromosomes [30, 31] or after stripping histones from DNA [32, 33] but, again, these might be preparative artefacts. The kinetics of nuclease digestion of conventionally prepared rat liver nuclei are also consistent with a looped structure; progressive detachment of DNA leaves a resistant fraction, presumably the base of the loop, attached to a pelletable structure [34].

Supercoiling also provides evidence for looping. Supercoils are maintained in DNA circles but not in broken or linear molecules. In 1973 it was suggested that linear eukaryotic DNA was supercoiled by organisation into loops and that differential supercoiling underlay differential gene expression [35]. Supercoiled DNA has distinctive properties [36]; these are shared by 'nucleoids' isolated by stripping off histones with 2 M NaCl [37]. Both sediment biphasically in gradients containing intercalators [37–42] and they bind ethidium [43], denature [43] and change shape [44, 45] similarly. Electron microscopy of spread nucleoids reveals supercoiled fibres attached to a collapsed nuclear 'cage' (Fig. 2b, c) [46, 47], suggesting that this linear DNA is looped and attached. Supercoiling in the loop stabilises any right-handed interwound superhelix at its base [43]. Supercoils arise because dissociation of nucleosome cores in 2 M NaCl leaves their imprints in DNA if it is looped [48, 49]. The DNA of nuclei isolated using hypotonic conditions also melts at the temperature characteristic of circular DNA [50, 51].

Loop size

Loop sizes have been estimated at 10–200 kb using these rather unsatisfactory preparations [12, 32, 34, 37–39, 43]; they change little during the cell cycle [52]. Recent measurements using nuclease digestion of HeLa cells extracted using physiological conditions give an average of 86 kb (Jackson, D. A., Dickinson, P. and Cook, P. R., unpublished results), suggesting that there might be one transcription unit per loop.

Specific attachments

Sequences attached to the skeleton can be mapped by progressively detaching DNA with a nuclease; sequences close to attachment sites should resist detachment and so be enriched in a pelleted fraction: those lying further away should be depleted. If attachments are generated artefactually, there should be no specificity in the aggregate and any given sequence will neither be enriched nor depleted. In fact, α globin sequences in HeLa nucleoids can be enriched eightfold whereas β and γ genes are depleted; α globin must lie closer to the attachment site than β or γ [53]. This 'detachment mapping' has been extended to many different preparations (e.g. matrices and scaffolds), but with variable results [5].

Attachment sequences can also be selected by incubating nuclei with DNA fragments to see which bind specifically. Little specificity is seen unless nuclei are first extracted with 2 M NaCl or heat-shocked and treated with lithium diiodosalicylate; then fragments containing enhancers or topoisomerase II sites bind specifically [54–60]. It is difficult to know whether such complexes are analogous to those *in vivo* or artefacts due to aggregated topoisomerase trapping its consensus sequence.

It seems prudent to leave this evidence until controversies are resolved, especially bearing in mind the unphysiological conditions used. It is hardly decisive evidence in favour of looping *in vivo*.

Loops and supercoils in native chromatin

Whether unrestrained supercoils (and, by inference, loops) exist in native chromatin is important because different loops might contain different degrees of supercoiling and these differences might affect transcription. Bases in DNA are buried and can only be read by unwinding the duplex. Supercoiling around the nucleosome is of opposite sense to that of the double helix so its torsional energy is potentially available for unwinding. Can this energy assist eukaryotic polymerases *in vivo* and do enzymes that alter supercoiling (i.e. topoisomerases) influence transcription? Such questions provoked the suggestion that nuclear DNA might be supercoiled [61] and lie behind the continuing search for torsionally strained or 'dynamic' chromatin. Early studies showed conclusively that supercoiling stimulates transcription *in vitro* [62, 63]; whether it does *in vivo*, and whether some chromatin is torsionally strained, are now the issues.

Chromatin containing free energy of supercoiling should bind more of an intercalator like psoralen than the sample when relaxed; extra psoralen binding was found in living bacteria but not in human and *Drosophila* cells [64, 65]. However, unrestrained supercoils in a eukaryotic chromatin fraction, perhaps the functional fraction, might have gone undetected by this insensitive method.

Various workers have claimed to have discovered such a minor fraction of 'dynamic' chromatin [66–74], but the evidence is at best only correlative, sometimes irreproducible [75–78] and, at worst, flawed. Some depends on the isolation of a minor fraction [66, 67], but accidental histone loss will inevitably introduce free supercoils. Other evidence involves novobiocin, which is assumed to inhibit topoisomerase II specifically, but it also inhibits RNA polymerases I, II and III directly [79], affects RNA attachments [80] and is used at such high concentrations that histones precipitate [81, 82] and oxidative phosphorylation is inhibited [83, 84]. In other experiments [85], topoisomerase I was injected into oocytes at concentrations equal to the enormous pool of free histone [86]. Such evidence is justly treated sceptically [76], but does not disprove the existence of a minor fraction containing free supercoils.

Recently, better but only circumstantial evidence has come from yeast cells with mutant topoisomerases and an appreciation that supercoiling is the inevitable consequence of transcription.

Topoisomerase mutants

Transcription of closed loops or circles poses topological problems that apply equally to models involving mobile polymerases or mobile templates [87–90]. Consider a 1-kb plasmid, transcribed by the polymerase in *Escherichia coli*. If the 'text-book' model were applied strictly, the polymerase (radius 7.5 nm), plus nascent transcript, attached ribosomes (each with radius 15 nm) and nascent protein would all track along a helical path, passing through the centre of the circular template (radius 9 nm if condensed by supercoiling) on transcription of each helical turn (i.e. every 10 bp). If this miracle were possible, the resulting transcript would be intertwined around the template once for every helical turn transcribed and could only be untwined by passing one of its ends through the circle, again once for every turn transcribed. Of the formal solutions, the likeliest involves no net rotation of polymerase and transcript about the helical axis, simply because they are too bulky. Then, the template must rotate, becoming posi-

tively supercoiled ahead of the polymerase and negatively supercoiled behind [89]. This compensatory coiling will quickly limit transcription unless removed by topoisomerases.

Various results show that topoisomerases do indeed play a central role. Topoisomerase I is closely associated with genes transcribed by polymerase I and II [91–105]. When the locus for topoisomerase I is inactivated, yeast cells remain viable [106–108], presumably because topoisomerase II rescues them. Topoisomerase II is indispensable as mutations in its gene are lethal [106–108]; although its main role in eukaryotes may be in replication and chromosome segregation [109, 110], it nevertheless forms 'open' transcription complexes [111].

Recently, Liu and Wang [89] suggested that transcription changes the superhelical density of bacterial DNA if positive and negative supercoils are not removed equally. As negative supercoils are relaxed only by topoisomerase I and positive supercoils only by topoisomerase II, supercoils of appropriate sense should accumulate if either enzyme is inactivated using inhibitors or temperature-sensitive mutants. In fact, positive supercoils accumulate on transcription of a test plasmid when topoisomerase II is inactivated [112]. In eukaryotes, both topoisomerases I and II relax positive and negative supercoils so their hypothesis is more difficult to test. Nevertheless, plasmids isolated from yeast cells lacking topoisomerase I are more negatively supercoiled than their less-transcribed counterparts [113–115]. Furthermore, plasmids become positively supercoiled when transcribed in specially engineered yeast expressing bacterial topoisomerase I but not mutated yeast topoisomerases I and II [115]. Positive supercoils also accumulate on transcription of circular DNA *in vitro* in the presence of topoisomerase I [116].

Interpretation of these experiments depends on complete inactivation of topoisomerases and on there being no other activities. This is not an academic reservation as bacterial topoisomerase mutants usually have compensatory mutations elsewhere [117–119]. Note also that they involve 'minichromosomes' whose behaviour may not be representative of chromosomal DNA. Furthermore, the existence of supercoils or torsionally strained chromatin does not necessarily prove the existence of loops. Supercoils might persist locally because their rate of diffusion to the place where they could be lost, chromosome ends, might be too slow. Slow diffusion might be expected down a long chromatin fibre, non-specifically snagged with other fibres in the dense tangle that is chromatin. So, again, the existence of loops *in vivo* still awaits formal proof.

EVIDENCE FOR ATTACHED TRANSCRIPTION COMPLEXES

Studies using 2 M NaCl

The first hints that transcription complexes might be attached came from studies on nucleoids prepared using 2 M NaCl [120] and so are rightly treated cautiously. However the central conclusions have now been confirmed using isotonic conditions (see below). Nucleoids have two advantages for this type of study: unlike matrices, they are prepared only by exposure to hypertonic salt concentrations (and not sequential treatments with both hypo- and hyper-tonic conditions) and their DNA remains supercoiled and unbroken. Therefore they have not accumulated artefacts due to exposure to hypotonic solutions, nor from binding nicked DNA [37].

If the text-books are correct, 2 M NaCl treatment, which dissociates pure polymerase from the template, should extract all pulse-labelled RNA from nucleoids: quite the opposite is found [44]. When the analogous experiment to Miller's is performed with nucleoids (i.e. DNA is spread) no nascent RNA is seen at the edges of the spread (Fig. 2c); all of it remains associated with a central skeleton (Fig. 2b) [47, 121]. Digestion with ribonuclease removed internal parts of nascent RNA but not 5′ caps, suggesting they were attached; they should be detached if the text-books were correct [121]. Another powerful control made non-specific aggregation unlikely: transcripts of an infecting nuclear virus, influenza, were also attached but those of a cytoplasmic rhabdovirus were not [122].

Transcribed genes were also attached [121]. In one extensive analysis the site of integration of a single integrated avian sarcoma virus or polyoma virus was 'detachment' mapped in various transformed clones [123]. In every case where the integrated virus was expressed, proviral sequences, particularly enhancers, resisted detachment. In some cases the transforming virus integrated into a site remote from an attachment point; then adjacent cellular DNA became attached, presumably through the provirus, but sequences on the other unaffected chromosome remained unattached. Some transformants spontaneously revert and lose the transformed phenotype; subsequently retransformed clones can be reselected following treatment with azacytidine. Integrated proviral sequences in the revertants, now non-transcribed, lost their close attachment, but regained it when reexpressed in the retransformants. These correlations with powerful internal controls provide strong evidence for an association of transcribed sequences with this skeletal structure.

As discussed earlier, other workers using superficially similar material obtained variable results so the interpretation of all remains controversial [5]. For example, transcribed regions of genes are rarely, if ever, attached to scaffolds isolated using lithium diiodosalicylate [12, 55, 124] (but see also [125]). But there is no reason to believe these results any more than the others, especially as they involve a hypotonic treatment and a mandatory heat-shock, which is known to induce aggregation [126–128].

Studies using 'physiological' conditions

It is difficult to know which buffer to choose when trying to reproduce physiological conditions because the precise ionic milieu *in vivo* remains unknown. There are also practical problems: the major cytoplasmic counterion is protein, an expensive additive to buffers, so Cl^- is commonly used, but this may reduce transcription rates to one tenth [21]. Notwithstanding such difficulties, it has at least now become possible to use isotonic salt concentrations, if not truly physiological ones, during isolation. Aggregation is avoided by first encapsulating cells in agarose microbeads of about 50 μm diameter [8, 129]. Agarose is permeable to small molecules, so cells can be regrown or extracted in a 'physiological' buffer containing Triton [130]; then most cytoplasmic proteins and RNA diffuse out to leave encapsulated chromatin surrounded by the cytoskeleton [8, 129, 130]. These fragile cell remnants are protected by the agarose coat, but accessible to probes like antibodies and enzymes.

To what extent is nuclear structure and function preserved? Heterochromatin, which decondenses readily when ion concentrations are altered, provides a marker for gross structural preservation: supercoiling, generated by sub-

sequent histone removal, provides another for molecular structure as a nick anywhere in a loop relaxes it. Both indicate that structure is preserved [130]. Function is also preserved. At least two different DNA polymerase activities can be demonstrated by lysing encapsulated cells. One is an aberrant soluble activity that uses nicked templates and is found irrespective of cell-cycle stage: it is the activity generally purified by biochemists. Another pellets with the microbeads and syntheses DNA *in vitro* at a rate equivalent to that *in vivo*: it is only found in S-phase cells and uses the native chromatin template [9, 131, 132]. Transcriptional rates are also well preserved. As rates *in vivo* are unknown, relative efficiencies cannot be determined but this preparation transcribes twice as efficiently as nuclei prepared conventionally and, as before, the activity resists extraction and uses the native chromatin template [130, 133, 134]. By these functional criteria, then, this preparation is as proficient in the vital nuclear activities as the living cell.

The two models for transcription can be distinguished by fragmenting chromatin with an endonuclease and removing any unattached material electrophoretically [133]. If the transcription complex is attached it should remain in beads: if unattached, it should electroelute from beads with most chromatin. Isotonic buffers can be used from cell lysis, through nuclease treatment and electroelution to subsequent polymerase assay. Removing 75% of the chromatin in this way hardly reduced transcriptional activity. Combined treatment with RNase and *Eco*RI, followed by electrophoresis, removed > 95% of nascent RNA (and so RNP) and 73% of DNA (and chromatin) but only 30% of the activity. A slight reduction in activity might be expected as the template has been cut into 10-kb pieces, so some genes are inevitably truncated. Clearly little, if any, activity escapes with chromatin, degraded RNA and associated RNP. Nascent RNA and the transcribed template also resisted electroelution [133].

These results are simply explained by an attached polymerase; nevertheless other possibilities should be excluded. The transcription complex cannot fortuitously have no net charge and so be unable to electroelute as the same result is obtained at a different pH [130]. If the complex is unattached, it must be so large that the polymerase is effectively attached. Even if it has a structure like a 'Christmas tree', RNase removes all 'branches' (i.e. RNP) and the 'trunk' has been cut into pieces very much smaller than the 150-kb fragments that can electroelute as chromatin [133]. In addition, transcribing 'minichromosomes' only a few thousand residues long also resist electroelution (unpublished work).

NUCLEOSKELETONS

Molecular nature

Unfortunately the molecular nature of any nucleoskeleton remains controversial. Structures isolated using 2 M NaCl (e.g. matrices, scaffolds, nucleoid cages) are probably derived from it but which parts are true constituents and which are artefactual additions is unclear [5]. To cite but one example: hypotonic conditions used to isolate nuclei convert pure RNP particles into fibres that cannot be redissolved in 2 M NaCl [10] so it is hardly surprising that RNP skeletons can then be seen.

Studies using the 'physiological' buffer provide a strong candidate for a nucleoskeleton that might exist *in vivo* [135]. When encapsulated cells are lysed using Triton, treated with *Hae*III and most chromatin electroeluted, electron microscopy of thick resinless sections then reveals a diffuse skeleton which ramifies throughout the nucleus (Fig. 3). Individual elements are about 10 nm wide with the axial repeat characteristic of intermediate filaments [136]. In view of the history of artefacts, images such as those in Fig. 3 must necessarily be treated cautiously, however appealing their structure. Nevertheless, if such a skeleton is an artefact formed prior to fixation, its creation cannot interfere with replication and transcription which continue at, or close to, *in vivo* rates. If an artefact is created on fixation, it is difficult to see why a diffuse network and not an aggregate is formed. Obviously it is important to demonstrate whether active polymerases are associated with this skeleton and whether other cytoskeletal elements like actin and tubulin, which cosediment with various subnuclear structures [7], also extend into nuclei and are associated with different functions.

'Miller' spreads

If a nucleoskeleton is composed of intermediate filaments, it is easy to explain why no skeleton is seen in 'Miller' spreads; some intermediate filaments are soluble in hypotonic solutions [137] so the skeleton might dissolve, leaving the 'Christmas tree'. Alternatively, spreading might strip the complex from the skeleton. Why, then, are skeletons not seen in conventional sections, especially those from highly active material isolated prior to fixation in 'physiological' conditions [31]? Perhaps they are too thin to allow visualisation of diffuse skeletons.

Relationship with other skeletons

Ribosomes (diameter 30 nm) pass through densely packed chromatin to the cytoplasm which has diffusional pores only 100 nm wide [138]. This makes it likely that they travel along tracks to their destination. Intermediate filaments could well provide such tracks as they form a nucleoskeleton, lamins [139–141] and the more familiar cytoskeleton, from nuclear pore to cell membrane [142, 143]. Then mRNA would remain attached to members of this one family at all stages of its life-cycle [121], from synthesis to translation [144] (but see [145]).

Targeting

Connecting genes physically with specific cytoplasmic destinations allows mRNA transfer along the connecting filaments to specific places; messages and the proteins they specify are indeed localised in the cytoplasm [146–149]. As nuclei rotate *in vivo* [150], interactions between cytoplasmic and nuclear filaments must be dynamic. Perhaps all nucleofilaments lead ribosomes to the nuclear periphery; then, rotation allows cytofilaments to be scanned so specific ones can be selected for ribosome transhipment.

Such connections also allow information flow from membrane to gene. Contacts between cell membranes generated during differentiation might stabilise underlying cytoskeletons, and hence nucleoskeletons, which in turn could influence gene expression. In this case it is the structure, rather than a second messenger, that transmits the information.

Duplication of the skeleton

If skeleton and attached DNA are duplicated simultaneously rather than separately, the replication site is also a nucleoskeleton-assembly site. This raises the possibilities that

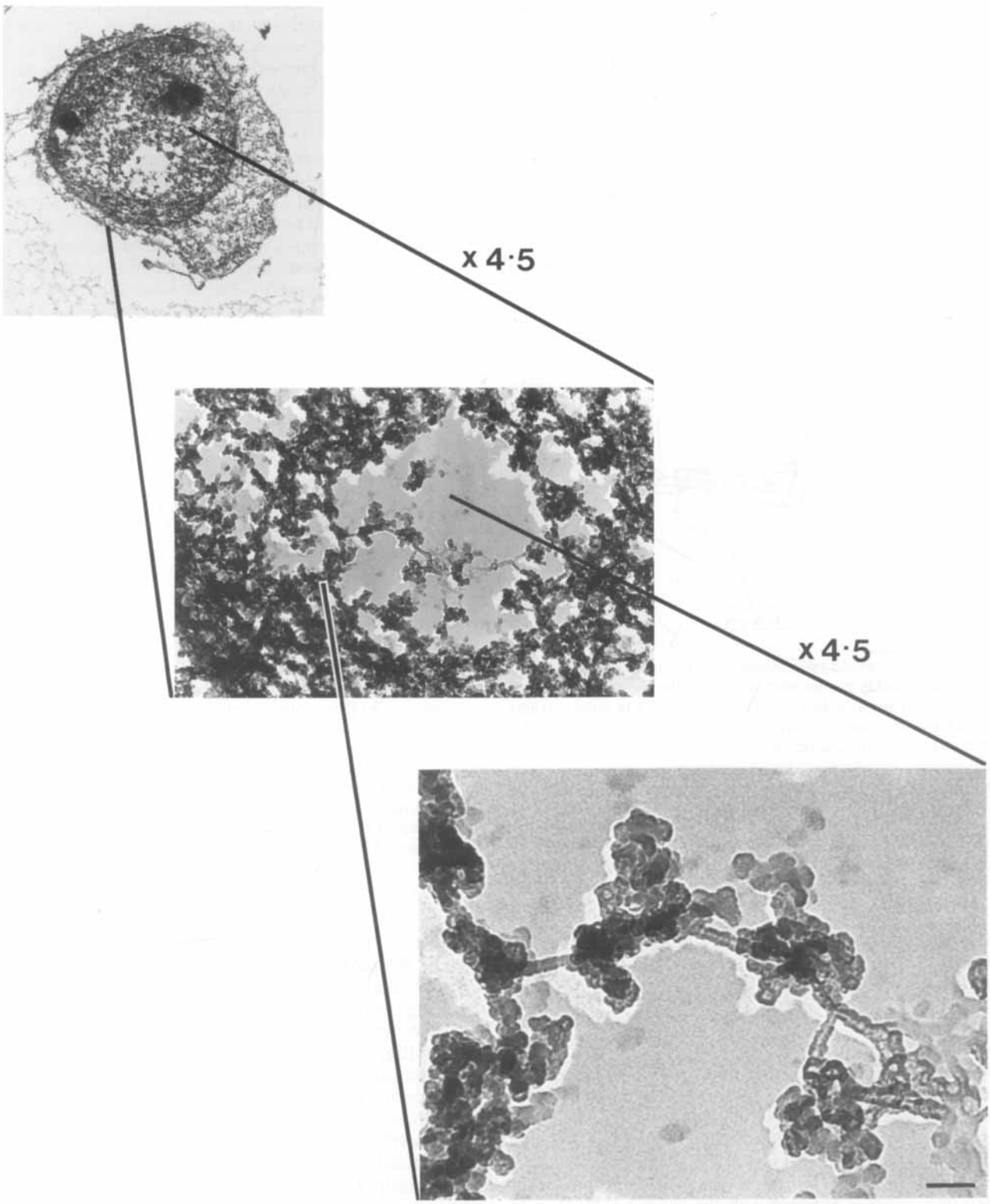

Fig. 3. *A candidate nucleoskeleton.* Electron micrographs at 4.5× higher magnifications of a thick resinless section of a HeLa cell derivative. Cells were encapsulated, lysed, obscuring chromatin cut with *Hae*III and then removed by electroelution. All procedures up to fixation took place in a 'physiologial' buffer. At the lowest magnification, the cell remnant can be seen surrounded by agarose and at the highest, residual chromatin clumps attached to a skeleton. The bar is 100 nm. See [135]

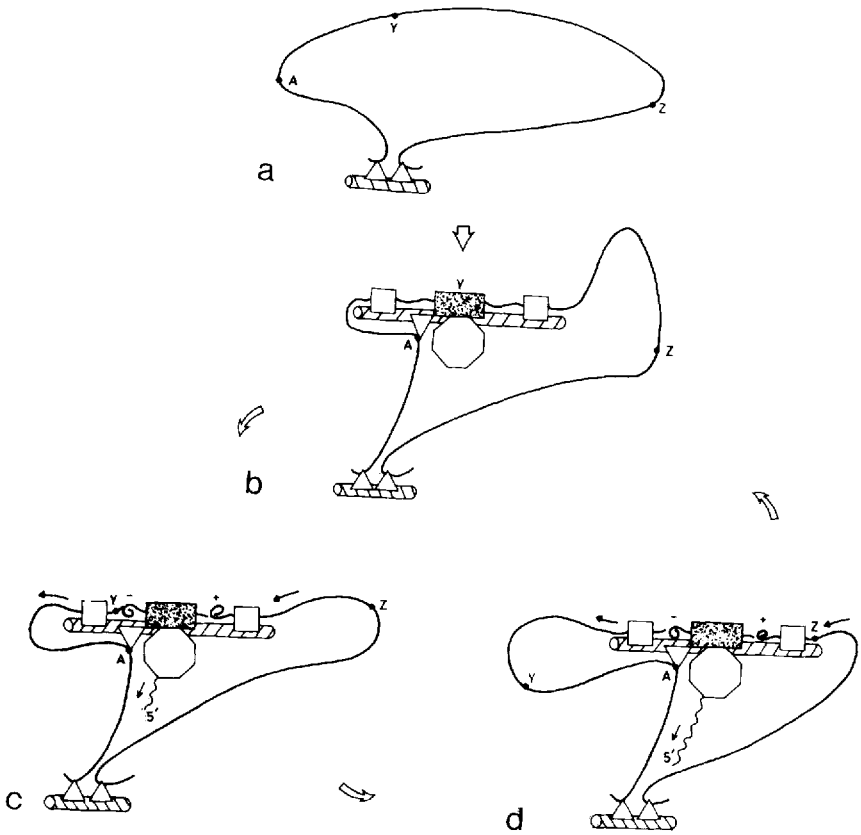

Fig. 4. *A schematic model for transcription (not to scale).* (a) A loop of DNA is shown attached to the skeleton (rod) at two sites (△). These attachments probably persist whether or not the loop is transcribed or replicated. The gene (Y−Z) out in the loop cannot be transcribed as it is remote from any attached polymerase. A marks an upstream activating sequence. (b) During development, the gene is activated by binding to the skeleton and assembly into an attached transcription complex containing polymerase (stippled rectangle), upstream binding sites (▽), topoisomerases (small squares) and RNA processing site (octagon). For the sake of simplicity, the complex is assembled on an additional skeletal element; transcription factors and another loop formed by an enhancer are also excluded. The upstream binding site now permanently tethers the gene to the complex and abuts the polymerase so that they can inter-communicate through physical contact or indirectly through variations in supercoiling of the connecting loop. (c, d) After initiation, DNA moves (arrows) through the complex and RNA (wavy line) is synthesised and processed. Probably the 5′ RNA end is attached [121] and a loop of RNA is extruded, rather than as shown. Positive and negative supercoils appear transiently as shown but are removed by topoisomerases. After the transcript is completed, A remains attached so the gene can easily return to its position in (b) and reinitiate synthesis

specific attachments, and so specific functions, might be inherited by the two progeny structures and that both might be replicated semi-conservatively.

Motors

Whichever model for transcription proves to be correct, it seems likely that additional motors drive the contortions of template and transcript. Actin is an obvious candidate; it copurifies with ribonucleoprotein complexes [151] and polymerase II [152], it is a known transcription factor [153] and injection of anti-actin antibodies into living newt cells inhibits transcription of lampbrush chromosomes [154].

A MODEL FOR AN ATTACHED POLYMERASE

A nucleoskeleton, a still ill-defined structure, is the structure to which the polymerase and associated activities are attached. These include transcription factors (e.g. those bound to upstream and downstream sites like enhancers), topoisomerases and processing enzymes (e.g. those involved in capping, splicing, methylation and polyadenylation). Some are tightly bound, for example > 95% of RNA polymerase II pellets with nuclear fragments [18] and little is displaced even by 600 mM $(NH_4)_2SO_4$ [8]; others (e.g. TFIIA) are less tightly bound [155]. The whole complex must be very large, dwarfing the template and associated proteins. Transcription occurs as the template passes through the polymerisation site; the resulting transcript remains attached during subsequent processing and transfer to the cytoplasm.

A specific model is illustrated in Fig. 4. A gene in a loop of DNA is initially remote from the polymerase and cannot be transcribed. On activation, it attaches to the skeleton and is assembled into a transcription complex. The DNA at the polymerisation site can be imagined as being connected to adjacent sequences through two ball-bearing races, topoisomerases, that allow it to rotate. These topoisomerases are drawn spatially separated from the polymerase, but may well abut it and consequently few supercoils will normally accumulate.

As the DNA moves past the polymerisation site it rotates so that the transcribed base on the helical template strand maintains the same topological relationship to the skeleton (Fig. 5). Template movements are analogous to those of a bolt driven through a fixed nut using a ratchet screwdriver (Fig. 5, below). The nut 'sees' the whole length of the thread as it passes through; the fixed polymerase 'sees' the transcribed

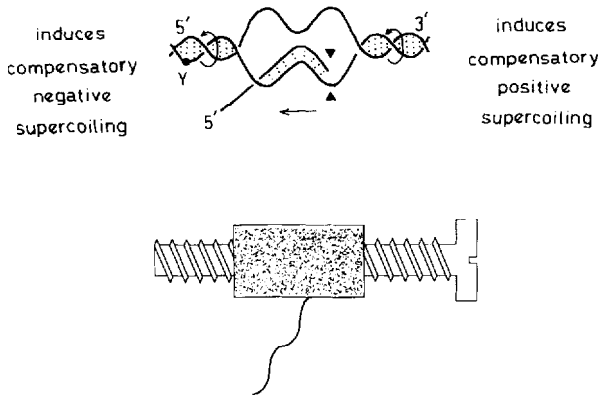

Fig. 5. *DNA movements at the polymerisation site.* DNA moves through the fixed polymerisation site (above) like a screw through a fixed nut (below). Above: Y is the first base to be copied. DNA moves to the left (arrow) and spins (arrows) so the transcribed base between the triangles always retains the same stereochemical relationship to the page (i.e. the skeleton). RNA is synthesised and extruded downwards to the left. Rotation induces compensatory supercoils to accumulate. Below: the bolt (DNA) rotates and passes leftwards through the fixed nut (polymerase). The wavy line shows the analogous position of the transcript

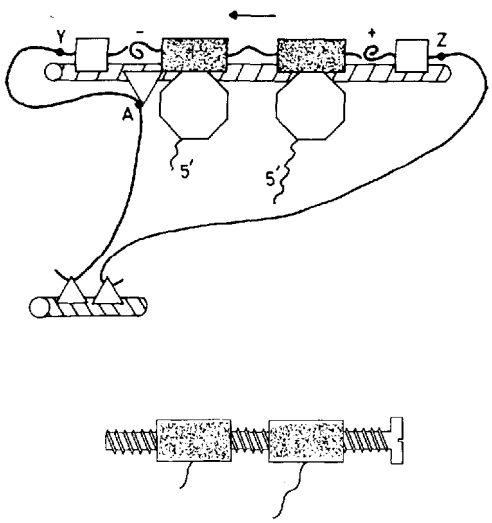

Fig. 6. *A transcription unit containing two polymerisation sites with the analogous bolt and two fixed nuts below.* Extra sites can be added to the right. Supercoils do not arise between sites if DNA moves through them at the same speed. Removal of the skeleton, perhaps during the preparation of a 'Miller' spread, yields the conventional view of a transcription unit. Symbols as for Figs 4 and 5

strand in the same way. As a right-handed twist drives the bolt, a right-handed twist accompanies DNA translocation and just as spinning the ratchet relieves wrist-strain, so a topoisomerase spins the DNA to release accumulated supercoils. The template is truly dynamic.

Highly active transcription units would contain additional polymerases attached to the right-hand side of the complex (Fig. 6). The template necessarily passes through them at the same speed so its axial rotation at each is identical and no supercoils build up between. The analogy here is that of one bolt being driven through two fixed nuts (Fig. 6, below); no topological problems arise within the bolt, only at its ends, so topoisomerases would be needed there. Intriguingly, topoisomerase I cuts are concentrated at the ends of the ribosomal locus [93, 94, 100, 105].

AN ATTACHMENT HYPOTHESIS FOR GENE ACTIVATION

In higher eukaryotes in which only a fraction of DNA is transcribed, most genes will be remote from the skeleton and so remote from polymerases. Sequences out in the loop will only be transcribed if they first attach (Fig. 7). Genes are switched on and off during development by attachment or detachment and cells in different tissues possess different arrays of attachments.

Some evidence supports this. Of different cell types (e.g. fibroblasts, lymphocytes, hepatocytes, teratocarcinoma cells from man, mouse, bird and insect) the only ones that fail to yield superhelical DNA and a nucleoid cage on lysis in Triton and 2 M NaCl are those that are transcriptionally inactive, i.e. mature hen erythrocytes and human sperm [38] (and unpublished work). Furthermore, when hen erythrocytes are fused with growing cells, they begin transcribing again as a matrix reforms [156]. And as described earlier, a sequential inactivation and reactivation of integrated pro-viral genes correlates with their detachment and reattachment [123].

What triggers specific attachments of target sequences during development? It could involve selective changes in chemical constitution (e.g. by methylation [157]), conformation (e.g. coiling or supercoiling in a different sense or degree [61, 158]) or binding of specific activators or repressors [159]. As all sequences associate transiently with the skeleton during replication (see later), this might be a prerequisite for transcriptional attachment [160–163]. Consider the α and β globin clusters which are probably each in one loop [53, 164]; genes in both clusters are arranged along the chromosome in order of their expression during development [165]. As the

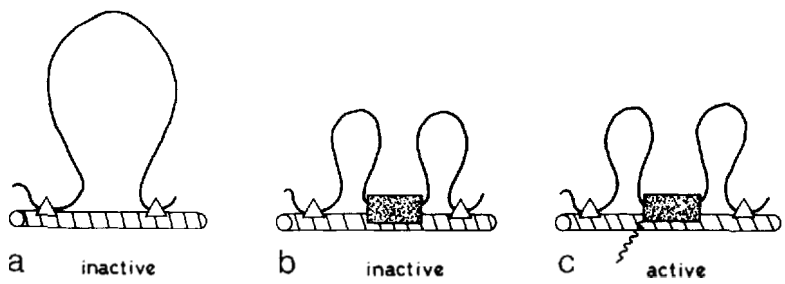

Fig. 7. *The attachment hypothesis.* A gene in a loop can only be transcribed when it attaches to the polymerase (stippled rectangle) at the skeleton. For example, the globin loop is shown inactive in a fibroblast (a). It attaches and becomes potentially active (but not expressed) in an erythroid stem cell (b), and expressed after addition of the appropriate transcription factors in an erythroblast (c). Symbols as for Fig. 4

first gene in the complex is replicated it becomes attached and so expressed. This attachment, and consequent expression, is retained subsequently and interferes with the transcriptional attachment, and so expression, of adjacent genes (see below). On replication later in development it detaches and the next gene along the chromosome attaches and it in turn becomes expressed. In this way gene order determines the sequence of expression during development.

This hypothesis requires that transcriptional attachments are sufficiently stable to survive when the template is replicated. The remarkable patterns seen in 'Miller' spreads of transcription units on sister chromatids supports this; the two patterns are similar, sometimes with the same transcript-free gaps in their middles [166]. This is explained with difficulty by the conventional model, by assuming two sets of diffusible polymerases somehow initiate together on the two units. However, such patterns are the inevitable consequence of transcription by attached complexes which are duplicated along with the DNA.

The inheritance of specific attachments might be allied to the inheritance of specific structures within loops [35, 61]. Consider the globin sequence in two different cells, with the same attachments but differing degrees of supercoiling. If during replication, detachments and net rotation of DNA are prevented, it is an inevitable consequence of semi-conservative replication that daughter loops inherit the superhelical density of parent loops. Then, structures of DNA (as supercoiling) or its attachments would contain all the heritable epigenetic information needed to trigger differentiation.

CONSEQUENCES OF THE ATTACHMENT MODEL

Loops, supercoils and topoisomerases

Ptashne [167] has recently explained various *cis* effects by a template looping that brings together upstream sites and the promoter. Such a looping has been incorporated into the specific model presented in Fig. 4. Indeed, the two models converge when the complex bridging the two sites becomes sufficiently large.

Loops also enable differing superhelical densities to be maintained locally and these differences could impinge upon transcription at a number of different points. In bacteria, supercoiling, whilst having little effect on transcriptional elongation [14, 63], has complicated effects on initiation [168]; the free energy of supercoiling often aids promoter 'opening' or unwinding. There is every chance that it also affects initiation in eukaryotes [169–173]. However, it is not yet clear how nucleosomes influence transcription; although they inhibit initiation *in vitro* [22–24, 174], they might do so by steric blocking or by competing for the free energy [175]. Their dissociation would release free energy of supercoiling to assist opening but whether they do so is controversial [176–178]. It is worth noting that the supercoils that accumulate ahead of the transcription site have the opposite sense to those in the nucleosome and so might destabilise it.

Recent models for transcription assume that topoisomerases will be soluble like the polymerase, diffusing to their site of action and removing supercoils as they arise [89]. However, if the polymerase is attached, then it seems likely that topoisomerases are too (Fig. 4). Topoisomerase II is closely associated with nuclear matrices and scaffolds [179–182] and activity is not extracted from encapsulated cells by Triton and isotonic salt concentrations (unpublished work). In addition, if topoisomerase were part of a larger complex, it would probably act at sites determined by three-dimensional structure; if freely diffusible, it would act randomly. In fact, topoisomerase I cuts are sometimes localised to only one strand [101].

Stable transcription complexes

The model requires that the template is stably attached to the skeleton and associated polymerases. Indeed, DNA forms stable complexes with polymerase I [183–185], II [24, 155, 186–189] and III [190, 191] and their stability does not depend on transcription *per se* [155, 188]. For example, when the U2 snRNA gene is injected into oocytes, it sequesters transcription factors so templates added subsequently cannot be transcribed. Remarkably, this complex is stable even after the polymerase has moved away from the initiation site and when transcription is inhibited with α-amanitin [192]. It is difficult to imagine how a diffusible polymerase could return to compete efficiently at the same initiation site but this is inevitable if the gene is attached. In Fig. 4, sequence A tethers the gene so that it remains permanently associated with a particular polymerase.

Capping, methylation, splicing and polyadenylation

Nascent RNA is capped [193, 194], methylated [195], spliced [196] and polyadenylated [197] before the polymerase completes synthesis. Therefore a model involving a mobile polymerase requires that all associated processing activities are dragged along the template. This seems unlikely as they are so bulky; it seems inevitable that the smaller template moves relative to them. For example, 'spliceosomes' are 40–60 nm in diameter and contain >50 different polypeptides [198] and approximately every 500 bases of heterogeneous nuclear RNA is complexed with >36 polypeptides [145]. In addition, heterogeneous nuclear RNA is associated with nuclear matrices [199] but the status of this association is controversial, especially bearing in mind that pure RNP particles aggregate in the hypotonic conditions used to prepare them [10].

The fact that *in vitro* systems splice [200, 201] and methylate [202] added RNA might be taken as evidence that no larger structure is involved. However, these involve crude extracts which, like *in vitro* transcription systems, always require preincubations when large complexes may form on added RNA. Conversely, there is no temporal lag if some skeletal structure is maintained. Globin pre-messenger is associated with matrices isolated from HeLa cells transfected with plasmids containing the rabbit β-globin gene. When such matrices are incubated with a splicing extract and ATP, the amount of matrix-bound pre-messenger falls and free intron lariat increases, without any lag [203]. Thus it seems likely that these post-transcriptional processes are all associated with the skeleton.

Stereochemical consequences

A symmetrical molecule like DNA can, in principle, bind to the polymerase in one of two orientations; some asymmetry must tell the polymerase which way to transcribe. A larger asymmetric structure would orient a symmetric polymerase correctly if it bound DNA at three or more sites [204]. Perhaps this explains why transcription units have at least three sites essential for initiation (e.g. the TAATA box, UAS and initiation site).

A number of general stereochemical consequences stem from the inflexibility of chromatin and the inability of the polymerase to diffuse freely; whilst certain attachments remain, this ensures that some regions of the template can never approach the polymerisation site. A promoter in any loop will be sufficiently close to perhaps as few as one attached transcription complex; every site is restricted to transcribing only those genes within range. The dedication of polymerases to transcribe particular genes is the inevitable consequence of the model and makes it easy to imagine how stable patterns of expression might be established during development. Specific stereochemical consequences will now be discussed.

Position effects, enhancers and transvection

Position effects were initially discovered in *Drosophila* and result in gene activity being suppressed by an adjacent (or *cis*) chromosomal rearrangement involving heterochromatin [205]. Suppression can affect any translocated gene. It occurs early in development and does not necessarily affect all cells carrying the rearrangement, but the closer the gene is to the breakpoint in heterochromatin, the more likely it is to be affected. Once initiated, the suppression is inherited by progeny cells within the fly, so that gene expression in different clones of cells within one tissue may produce a variegated phenotype. Such position effects are now commonly found in 'transgenic' animals; whether the transgene is expressed depends on its integration site [206–211].

The inactivation of genes by position effects is explained with difficulty by models involving diffusible molecules but is naturally explained by structural ones [61]. Translocation of a hitherto active gene from its usual environment, which presumably contains the appropriate attachment sites and associated polymerases, into a heterochromatic region devoid of them, would inevitably inactivate it.

'Enhancers' provide examples of position effects at the molecular level. They increase expression of adjacent genes, but not those on other chromosomes; they may lie 5′ or 3′ to the initiation site and act over many thousands of base pairs [212, 213]. Their effects are usually explained in terms of 'entry' sites for diffusible polymerases, but if so the polymerase must then 'scan' thousands of base pairs both upstream and downstream for the initiation site. The attachment model sees enhancers as sequences that bind to the nucleoskeleton, bringing adjacent genes into close proximity to bound polymerases. Indeed, enhancers are the sequences most closely associated with nucleoid cages [123].

Recently, particularly powerful enhancers or 'dominant control regions' have been uncovered at the extreme ends of the β-globin locus [164]. These sequences, selected because of their hypersensitivity to nucleases, were used to construct a 'mini-locus' containing the β-globin gene; on introduction into mice, the transgene was expressed in a tissue-specific manner, independently of position. These sequences also bind to scaffolds [60] and presumably represent attachment sequences, isolating the β-globin gene from the effects of adjacent DNA in the chromosome by forming a loop.

If a fully active transcription complex is assembled at the nucleoskeleton from the promoter and distant *cis*-acting sequences by looping out intervening DNA, a promoter from one chromosome might occasionally be incorporated into a complex with a *cis*-acting sequence from another. Such an event would go undetected unless functional sequences on one chromosome complemented deficiencies on another and unless the complementing chromosomes were together. Just such an effect may underlie 'transvection' in the bithorax complex in *Drosophila* which depends on chromosome pairing [214, 215]. It has been explained in terms of nuclear messages with limited diffusional ranges or trans-splicing; recent experiments make such explanations unlikely [216]. The expression of *Ubx*, a gene in the complex, is regulated by *cis*-acting elements lying 40–60 kbp on either side. Some of these can regulate a second copy of *Ubx* on another chromosome, but only if paired with it. Perhaps a skeleton brings *cis*-acting enhancers from one chromosome together with the promoter from another.

Transcriptional interference

The phenomenon of 'transcriptional interference' is commonly found when two functional genes carried by retroviral vectors are inserted into a chromosome. Assay of the population shows both genes to be transcribed, but assay of individual cells or clones shows only one of the two promoters to be active [217–221]. Inactivation of one promoter improves expression of the other [222]. Such effects are also found with rearranged cellular genes [223] or transfected minichromosomes packed with transcription units [224, 225]. Transcriptional interference is usually interpreted in terms of mobile polymerases running from one transcription unit into another, but then it is difficult to see how transcription of a downstream gene might inhibit one upstream or why transcription of the upstream gene does not stimulate transcription of the downstream one. (Note that interference cannot be seen in most 'transient' and stable transfectants because cells generally contain > 1 plasmid.)

Interference is simply explained if stereochemical constraints determine how closely adjacent attached complexes can be spaced; only one of two adjacent promoters can attach at any one time and be active. Both would be activated by increasing the interstitial DNA above a critical minimum which must be > 5.2 kb [220]. Perhaps such interference normally controls expression of adjacent genes during development so that only one of them could be active at any one time [224]. Examples might include switching between the early and late promoters during viral growth [163] and between the *Adh* promoters during development in *Drosophila* [221].

Analogous stereochemical constraints should affect how closely processing sites can be spaced: sites on the transcript are usually further apart than this minimum spacing. Such interference might explain why the transcriptional machinery ignores a polyadenylation signal in the 5′ long terminal repeat of retroviruses and then uses the identical sequence further downstream in the 3′ repeat [226]. This has been explained by transcript looping [227], but could equally result from steric hindrance of any polyadenylation at sites too close to the polymerisation site or an attached 5′ cap. Again, increasing the separation between the cap site and the 5′ signal should allow polyadenylation and give some estimate of how far apart the two sites are in space.

Role of introns during transcription

Genes with introns are transcribed in transgenic mice at least tenfold more efficiently than their counterparts without introns [228]. As no sequence-specific signals have been detected in some of the introns tested, it seems that introns must play some general structural role. Just such general effects might be expected if the gene was looped and attached to a number of polymerisation sites; thus, in Fig. 6, intron loss

might make it impossible for a shorter template to loop back and attach to two polymerases simultaneously. Here, interference within a transcription unit, rather than between units, reduces the number of attachments and so the transcription rate.

Replicational interference

A replication fork would interfere with a transcription unit in an analogous way. Most transcription units in *E. coli* happen to be aligned on the chromosome so that the direction of replication and transcription are similar; it has been suggested that this results from evolutionary pressures to prevent polymerases colliding [229]. But if active DNA and RNA polymerases are both attached (see below) they cannot collide. Instead, passage of DNA during replication and transcription through the two complexes in the same direction would minimize interference; movement in opposite directions would be impossible so one process, presumably transcription, must stop.

Other polymerases and functions of DNA

This discussion has concentrated on the role of the nucleoskeleton with respect to transcription. However, the attachment model can equally be applied to other functions of DNA (i.e. replication, repair and recombination). Indeed, very similar kinds of evidence to that reviewed above, especially that derived using isotonic conditions, show that nascent DNA and the relevant DNA polymerases (α and β), if active, are associated with the nucleoskeleton [131, 132]. Activation is again seen as a binding of sequences to polymerases associated with a skeleton [230]. Structural models [231, 232] (but see [233]) would seem essential to explain how damage induced by ultraviolet light could be removed selectively from the transcribed, but not the non-transcribed strand, of the DHFR gene [234] and how adjacent replicons might initiate coordinately [235].

CONCLUSIONS

The 'text-book' model for transcription sees the polymerase and transcript moving along DNA unattached to any skeleton. Like many received ideas, this one seems to have little decisive evidence for it. Accurate transcription at *in vivo* rates by a soluble system would provide strong evidence, but existing systems are still very inefficient. Something that influences rates by a few orders of magnitude is clearly lacking. Therefore it seems worthwhile to examine an alternative in which the template moves past a polymerase attached to a nucleoskeleton; this nucleoskeleton is the active site. The best evidence for this alternative is circumstantial and stems from studies on cells fractionated using only one kind of procedure (lysis in a 'physiological' buffer, followed by nuclease digestion and electrophoretic removal of detached chromatin) and so must be corroborated. This crude system transcribes very efficiently. Obivously, analysis of such an insoluble polymerase–nucleoskeleton complex poses a difficult challenge to biochemists and formal proof of the alternative model will be difficult.

Our perception of whether template or polymerase moves is determined by our perception of their relative sizes. We now know that the polymerase and associated activities (including transcription factors, topoisomerases and associated splicing, capping, methylation and polyadenylation complexes) must dwarf the template; they bind to each other and to a number of sites on the DNA, forming it into loops. When that complex becomes sufficiently large, the two models inevitably converge.

If active polymerases are indeed stably attached, our DNA-centred universe becomes a skeleton-centred one. Enhancers bind to the skeleton, not the polymerase. Transcription factors bind to the skeleton as well as to DNA. Most importantly, as biochemists we look in the pellet rather than in the supernatant.

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