6. Transcription by immobile RNA polymerases

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Summary

An unstated assumption in current models for transcriptions is that a polymerase tracks along the template as it synthesizes RNA. However, experiments using 'nucleoids' derived by lysing cells in a non-ionic detergent and 2 M NaCl originally suggested that RNA was synthesized by a polymerase attached to an underlying nucleoskeleton. These experiments were subject to the criticism that the associations seen resulted from the artefactual aggregation of nascent RNA. Further experiments using more physiological conditions have confirmed the existence of an intermediate-filament-like nucleoskeleton and have shown that active polymerases resist electroelution from nuclei, presumably because they are attached to the skeleton. Whether immobilization affects polymerase activity has also been tested directly by attaching to plastic beads a pure enzyme that is widely used for transcription *in vitro*—the RNA polymerase of bacteriophage T7. Although initiation is inhibited, immobilization has no effect on elongation. It is suggested that genes become active by binding to an attached polymerase and then transcripts are generated as the template moves past the fixed enzyme.

Introduction

A highlight of my first year as a graduate student in 1967 were the lectures by Henry Harris in this theatre on the 'Nucleus and Cytoplasm' (Harris 1967). The house was packed, the atmosphere electric, and the delivery word-perfect. The lectures were perfused with a simple message: look at the evidence underlying accepted ideas; if it is unsatisfactory, there is an opportunity to spend 'many hours of simple pleasure' doing experiments to test those ideas.

Fig. 6.1 shows the front cover of the third, paperback, edition of the book that resulted from these lectures (Harris 1974). It illustrates heterokaryons formed by fusion of mouse A9 cells with chick erythrocytes, the subject of my research as a graduate student. The erythrocyte nuclei, initially highly condensed and transcriptionally inert, are swelling, decondensing their chromatin, and becoming transcriptionally active; only when nucleoli appeared within the reactivating nuclei were chick genes expressed. No underlying nucleoskeleton was visible in the light microscope in either kind of nucleus.

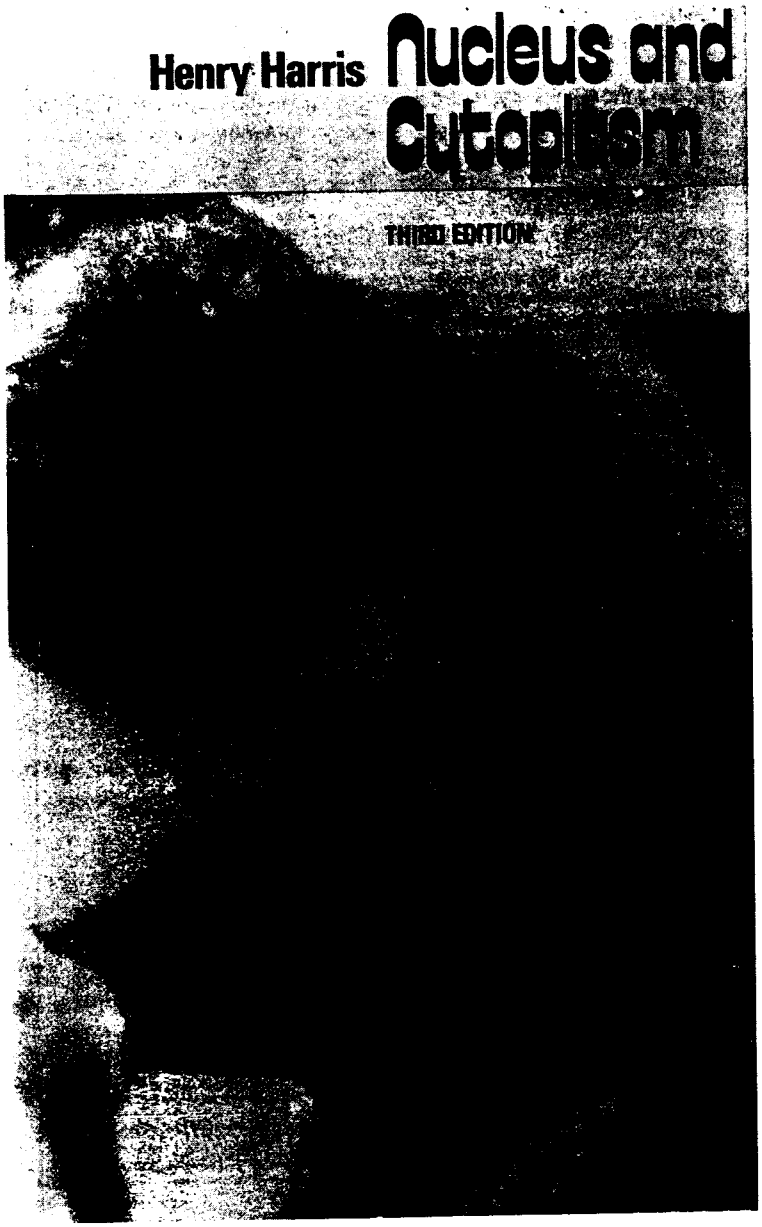

Fig. 6.1. The front cover of the 3rd edition of *Nucleus and cytoplasm*. (With permission of Oxford University Press.)

I worked on hypoxanthine-guanine phosphoribosyl transferase in these heterokaryons, which is encoded by the X-chromosome in mammals. It was only natural, then, that I should wonder what the basis of the inactivation of one of the two X-chromosomes in cells of female mammals might be; eventually I suggested that the linear chromosomes of higher eukaryotes must be organized into loops, and that differences in supercoiling in those loops underpinned differences in X-chromosome activity (Cook 1973, 1974). We then demonstrated that eukaryotic DNA was indeed supercoiled and organized into loops by attachment to a nuclear 'cage' and that supercoiling was lost as chromatin condensed during maturation of chicken erythroblasts into transcriptionally inert erythrocytes (Cook and Brazell 1975,

1976). Inevitably I asked the question: do RNA polymerases work out in the loop or at the base of the loop? I shall describe experiments that go some way to answer this question. But first, what is the evidence for current models for transcription?

The 'textbook' model for transcription

An unstated assumption in current models is that a polymerase tracks along the template as it synthesizes RNA (e.g. Alberts *et al.* 1983; Darnell *et al.* 1986). This assumption follows naturally from the relative size of the polymerase and template; presumably it is the smaller of the two that moves. But despite almost complete acceptance of the 'textbook' model, there seem to be only two kinds of evidence to support it.

The first kind is circumstantial; soluble polymerases work *in vitro* in the absence of any immobilizing skeleton. Why invoke any role for a skeleton, when we can mimic so well what happens *in vivo* without one? However, it is not widely appreciated that most RNA polymerase II in the cell is insoluble (Beebee 1979; Weil *et al.* 1979; Jackson and Cook 1985*b*). Of course, soluble enzymes are found in certain cases, for example in frogs' eggs, but they are inactive stockpiles, awaiting later use. Moreover, soluble polymerases isolated by most biochemists are inefficient and only become active when incorporated into large complexes. Thus, when cell extracts are incubated with appropriate templates, essentially all active RNA polymerases I, II, and III assemble into complexes that can be pelleted by a 5 min spin in a microcentrifuge (Culotta *et al.* 1985). Clearly, even these 'soluble' enzymes quickly form large complexes *before* becoming active. 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.

The second kind of evidence is provided by 'Miller' spreads (Miller 1984) and is apparently decisive. These spreads are prepared by dropping nuclei into a solution that is little more than distilled water (sometimes containing the detergent 'Joy'). Removing counter-ions charges chromatin, which expands and bursts the nucleus; individual chromatin fibres and beautiful 'Christmas tree' complexes can then be seen at the edge of the spread chromatin. No skeleton is visible. But, *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.

Active polymerases are attached to nucleoid cages

More than 10 years ago Shirley McCready did an analogous experiment to Miller's—she spread HeLa derivatives prepared not by reducing the tonicity, but by *increasing* it with 2 M NaCl (McCready *et al.* 1979). The now naked DNA, initially confined within a residual nucleoid 'cage', spreads to form a skirt that is attached to, and surrounds, the collapsed cage (Fig. 6.2). The DNA is supercoiled. Autoradiography showed that there was **no** nascent RNA in the skirt; **all** remained associated with the cage, which was presumably where it was made (Jackson *et al.*

Fig. 6.2. DNA is organized into loops containing supercoils by attachment to a nuclea 'cage'. HeLa cells were treated with Triton (to permeabilize cell and nuclear membranes) an 2 M NaCl (to strip histones from the DNA). The resulting 'nucleoid' has been prepared fo electron microscopy using Kleinschmidt's procedure. A tangled mass of supercoiled DN fibres extend from the 'cage' to the edge of the field. Bar: 10 μm. From Jackson *et al.* (1984)

1981). Dean Jackson also removed most DNA with nucleases and found that transcribed sequences, and especially enhancers, were amongst the minority of sequences that still remained attached (reviewed by Jackson *et al.* (1984)). This suggests that the cage was the site of transcription and allowed us to rationalize the results we had obtained with nucleoids derived from different cells of the erythrocyte lineage: erythroblasts yielded well-developed cages (associated with supercoiled DNA) and were transcriptionally active; erythrocytes gave no cage (so their DNA was relaxed) and were transcriptionally inert.

Encapsulated cells allow use of a physiological salt concentration

We used unphysiological conditions for these experiments (as does nearly everybody), because chromatin aggregates into an unworkable mess at isotonic salt concentrations. This, coupled to the fact that transcription complexes are very sticky, led to the suspicion that transcript–cage complexes were isolation artefacts (Cook 1988). Therefore we developed a procedure that allowed the use of more physiological conditions (Jackson and Cook 1985*a*; Jackson *et al.* 1988). Cells were encapsulated in agarose microbeads of about 50 μm diameter (Fig. 6.3, left). As agarose is permeable to small molecules, cells can be regrown or extracted in 'physiological' buffers containing Triton; then most cytoplasmic proteins and RNA diffuse out to leave encapsulated chromatin surrounded by the cytoskeleton (Fig. 6.3, right). These fragile cell remnants are protected by the agarose coat, but accessible to probes like antibodies and enzymes. Whilst one cannot be certain that any isolate is artefact-free, this type contains intact DNA and essentially **all** the replicative and transcriptional activities of the living cell. As the attachments that I will describe involve polymerases, it seems unlikely that they are generated artefactually when all activity is retained.

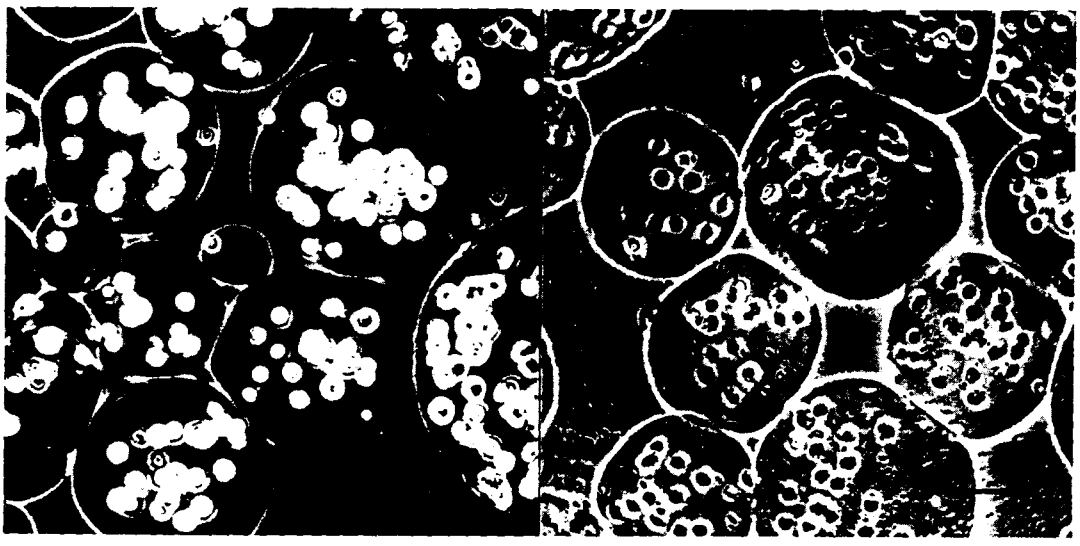

Fig. 6.3. HeLa cells encapsulated in agarose microbeads, before (left) and after (right) lysis with Triton.

Active polymerases are associated with a nucleoskeleton

We used the encapsulated and lysed cells to examine whether polymerases were attached to some large structure in the nucleus. Models involving mobile or immobile (i.e. attached) polymerases can be distinguished by fragmenting the encapsulated chromatin with an endonuclease and then removing any unattached material electrophoretically. If polymerizing complexes are attached to a larger skeletal structure, they should remain in beads: if unattached, they should electroelute from beads with most chromatin (Fig. 6.4). (Note that chromatin containing DNA fragments of 150 kb can escape from beads.) Cutting HeLa chromatin into <10 kb fragments, followed by electroelution of most chromatin, leaves residual clumps of chromatin associated with an intermediate-filament-like skeleton (Fig. 6.5; Jackson and Cook 1988). However, chromatin removal hardly reduces the activity of RNA polymerases I and II (Fig. 6.6) or DNA polymerase α; nascent RNA and DNA also

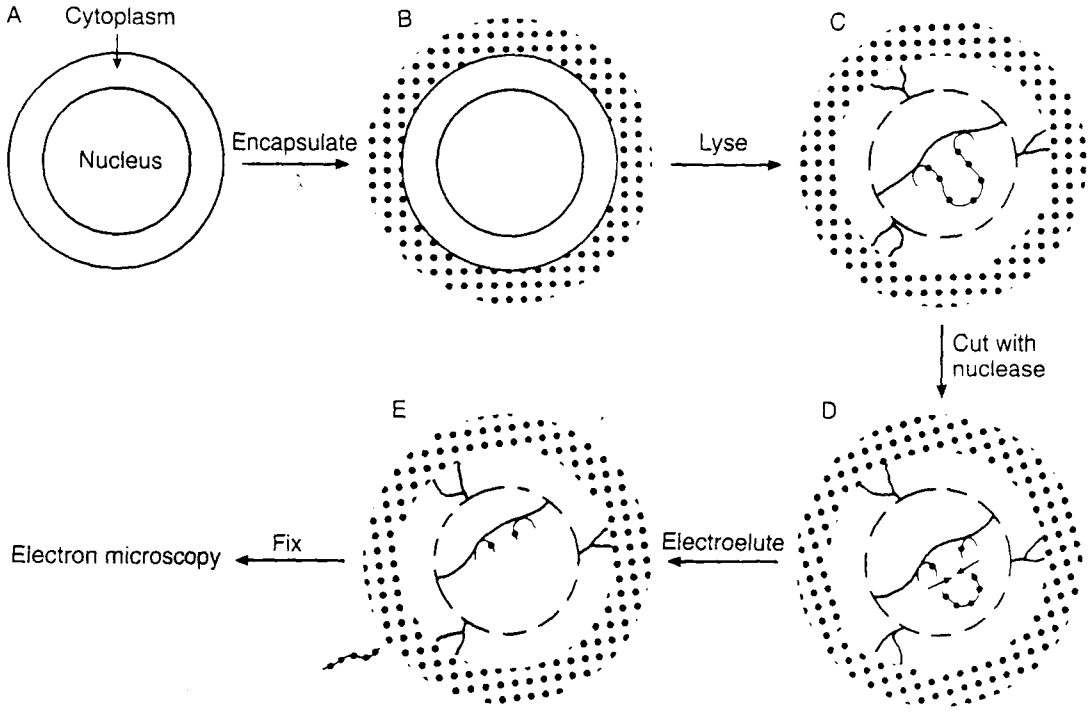

Fig. 6.4. Outline of experimental approach. Cells (A) are encapsulated (B) in agarose microbeads (stippled area), lysed (C), and washed in a 'physiological' buffer. Structures too large to escape through agarose are left in beads and include the cytoskeleton, nuclear lamina (dashed line) and chromatin (looped 'beads on a string') which generally obscures any underlying nucleoskeleton. Chromatin is fragmented (D) by addition of a nuclease (arrows) and small unattached pieces are removed electrophoretically (E). Finally, samples are fixed and viewed in the electron microscope; any underlying nucleoskeleton can now be seen in the relatively empty nucleus. Alternatively, attachments of polymerase can be analysed by comparing polymerizing activities in beads that have been subjected to electrophoresis or stored on ice. If the polymerase is associated with the skeleton, all activity should resist electroelution; if not, most activity should be lost with the electroeluted chromatin.

Fig. 6.5. Electron micrographs of thick resinless sections of encapsulated HeLa cells from which 80 per cent of the chromatin had been removed as in Fig. 6.4. (A) Low power, showing a section through a HeLa cell. The surrounding agarose cannot be seen at this magnification. (B) Medium power, showing the region in the square in (A) (the top left-hand corner is filled in for orientation). (C) High power showing residual clumps of chromatin still attached to a nucleoskeleton. The nuclear lamina runs across the top of the field. (From Jackson and Cook 1988.)

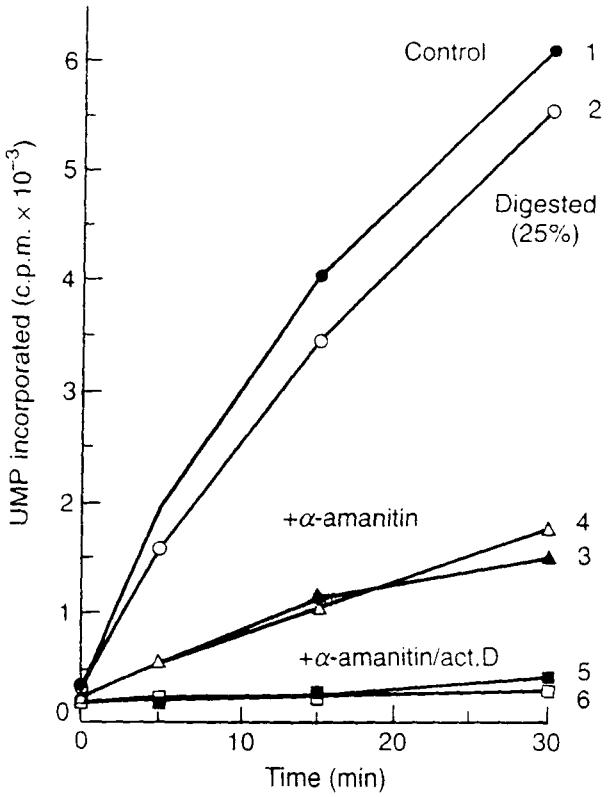

Fig. 6.6. Active RNA polymerases resist electroelution. Cells were encapsulated, treated with or without *Eco*RI, and detached fragments electroeluted as in Fig. 6.4. The time-course of incorporation of [^{32}P]UTP into acid-insoluble material by beads treated in various ways is shown. In some cases cells were treated with actinomycin D before harvesting, in others lysed cells were preincubated with α-amanitin before transcription. Curve 1: control, without inhibitors, digestion with *Eco*RI, or electrophoresis. Curve 2: without inhibitors, but digested and electroeluted (25 per cent of the chromatin remained). Curve 3: without digestion or electrophoresis, but with α-amanitin. Curve 4: with digestion, electroelution (25 per cent of the chromatin remained), and α-amanitin. Curve 5: without digestion or electrophoresis, but with actinomycin D and α-amanitin. Curve 6: with digestion, electrophoresis (25 per cent of the chromatin remained), actinomycin D, and α-amanitin. Despite the removal of 75 per cent of the chromatin, essentially all RNA polymerizing activity, which is mostly RNA polymerase II, is retained in beads (curves 1 and 2). The α-amanitin-resistant and actinomycin D-sensitive activity (i.e. RNA polymerase I) also resisted elution (curves 3 and 4). (From Dickinson *et al.* 1990.)

resisted electroelution, presumably because they are attached to the skeleton (Jackson and Cook 1985*b*, 1986*a,b,c*; Jackson *et al.* 1988; Dickinson *et al.* 1990).

After removing most chromatin (as in Fig. 6.4), the size of the loops can be deduced from the size of the residual attached fragments and the percentage of chromatin remaining in beads (Jackson *et al.* 1990). Loop sizes ranged from 5 to 200 kb, with an average of 86 kb; the smaller loops were probably the transcriptionally active ones. Loops in nuclei isolated by conventional methods, as well as matrices and scaffolds—which are all prepared in non-isotonic buffers—had smaller loops; many of their attachments of chromatin fibre to the skeleton must be generated artefactually during isolation.

Dean Jackson has recently gone on to map which sequences attach viral minichromosomes to the skeleton in transfected cells (Jackson and Cook, 1993). Non-transcribed minichromosomes in the population eluted from nuclei but transcriptionally active ones did not. Cutting the attached fraction with *Hae*III enabled most resulting ~400 bp fragments to elute and analysis of the residual fragments showed that no single sequence was responsible for attachment: rather each minichromosome was attached at only one or two points through a promoter or somewhere in a transcription unit (i.e. probably through an elongating RNA polymerase II). The latter attachments must change dynamically as the template slides past the attachment site. It is obviously tempting to extrapolate these results to cellular loops and suggest that they, too, are attached only by active polymerases and promoters (Jackson *et al.* 1992).

We have recently visualized sites of transcription by fluorescence microscopy (Jackson, *et al.* 1993). Encapsulated and permeabilized HeLa cells are incubated with Br-UTP to extend nascent RNA chains by ~500 nucleotides; then sites of incorporation are directly immunolabelled using an antibody against Br-RNA.~300 focal sites of incorporation (i.e. RNA synthesis) can be seen in each nucleus; most of these also contain RNA polymerase II and a component of the splicing apparatus detected by anti-Sm antibodies. α-amanitin, an inhibitor of RNA polymerase II, prevents incorporation into these foci so that ~25 discrete foci can be seen more clearly in nucleoli. All these fluorescent foci remain after removing ~90 per cent of the chromatin. As calculations show that each focus contains many transcription units, this suggests that an underlying skeleton must organize groups of transcription units (in both nucleolar and extra-nucleolar regions) into 'factories' where transcripts are both synthesized and processed. We will now visualize these factories by electron microscopy, much as we have done for the analogous replication factories (Hozák, *et al.* 1992).

The use of 'physiological' conditions and recovery of essentially all activity, rather than a minor fraction, make explanations of these results based on artefacts involving aggregated polymerases difficult to sustain. The polymerizing complexes cannot fortuitously have no net charge and so be unable to electroelute as the same results are obtained at a different pH (Jackson *et al.* 1988). If the complex is unattached, it must be so large that the polymerase is effectively attached. But the simplest interpretation is that active polymerases are attached.

The topology of transcription

If active polymerases are attached, presumably they are immobile. How, then, does transcription occur?

Transcription of a double helix poses various topological problems. One concerns templates with ends that are unable to rotate freely, for example if organized into circles or loops (Jackson *et al.* 1981; Liu and Wang, 1987). Another concerns the interlocking of template and transcript that results if the polymerase

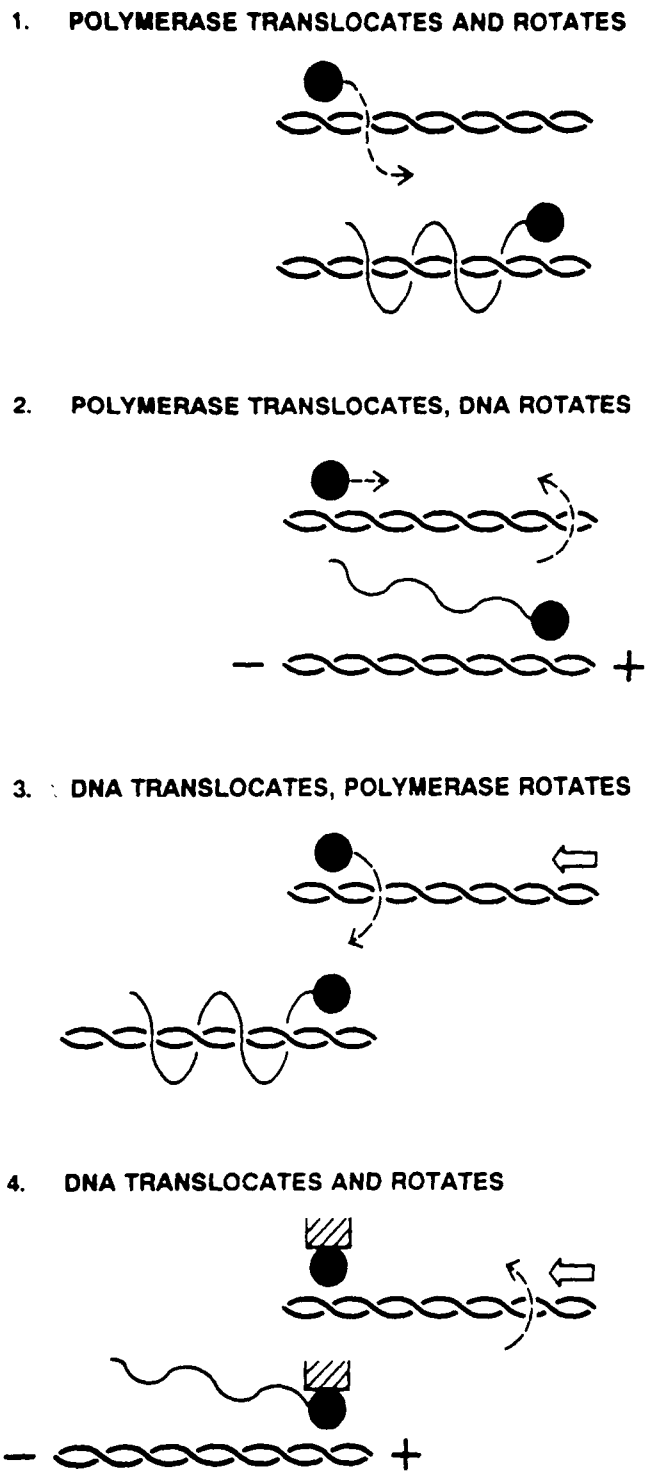

Fig. 6.7. Models for transcriptional elongation involving mobile or static polymerases (black circles) and double-helical templates. The upper figure in each model indicates initial relative positions; subsequent movements are shown by arrows. Lower figures show final positions after generation of transcripts (wavy lines attached to polymerases). + and – indicate the formation of domains of positive and negative supercoiling. In 4, the hatched area immobilizes the polymerase. (From Cook and Gove 1992.)

tracks along a helical strand, as in 'textbook' models. Polymerase and template must move relative to each other, both rotationally around the helix axis and laterally along it, so relative motions can be classified in four ways, depending on which of the two players (polymerase or DNA) performs which of the two movements (rotation or translocation).

The first model in Fig. 6.7 involves a mobile polymerase both rotating about and translocating along a static template. Then the polymerase, plus nascent transcript, must rotate about the template, once for every 10 bp transcribed. This gives a transcript that is intertwined about the template and we have no mechanism for 'untwining' them. This untwining problem seems insuperable, making model 1 unlikely. Model 3 faces the same intractable problem.

This problem is sidestepped if DNA rotates instead of the polymerase. In model 2—the 'twin-supercoiled-domain' model (Liu and Wang 1987)—the enzyme translocates laterally but its rotation is restricted, perhaps by the frictional drag of the

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Fig. 6.8. Immobilizing T7 RNA polymerase. (A) The structure of the plasmid encoding the hybrid polymerase. The sequence of the linker is shown below: the underlined ACT is codon 392 of *malE* (the gene for the maltose-binding protein), the region in bold encodes the protease Xa recognition sequence (IEGR), and the ATG codes for the first amino acid of the polymerase. (B) Transcription from P_{tac} and subsequent translation leads to the formation of a hybrid protein, with maltose-binding protein and polymerase domains, connected through a peptide linker containing the Xa-cleavage site, IEGR. (C) Cartoon of two hybrid proteins immobilized by attachment via antibodies directed against the maltose-binding moiety (Y-shaped structures) to protein A (circles) covalently attached to plastic (hatched area). The upper hybrid protein has bound template and generated a transcript (wavy line); the lower one is inaccessible to template. Treatment with Xa releases both polymerases. (D) Bound and free RNA polymerases elongate at equal rates. Elongation rates were measured under conditions where initiation was suppressed, both using heparin and by removing excess template. Hybrid protein was bound to beads and transcription initiated by adding ATP, CTP, and GTP, but not UTP. Then initiated complexes with 7–nucleotide-long transcripts are formed, as the first U is incorporated into nascent RNA at position eight. All samples were washed free of excess template, some were incubated for 3 or 30 min at 20 °C (–/+ heparin, –/+ Xa) and some were then rewashed to remove any detached polymerase. Transcriptional elongation was then re-started by addition of [α^{32}P]UTP. Equal volume reactions containing labelled transcripts were run on a denaturing gel and an autoradiograph was prepared. Samples were withdrawn at 0.25, 5, and 15 min (indicated by triangles), giving three tracks per reaction. Nucleotide sizes are indicated on the left. At the low UTP concentration used, transcription is inefficient and transcripts stall or terminate prematurely wherever UTP is required. For example, many do so 123 nucleotides downstream from the promoter, where four consecutive uridines are incorporated. Most transcripts synthesized after 3 min pre-incubation in the absence of heparin are shorter than 123 nucleotides (lanes 1–3). Heparin, by preventing reinitiation, suppresses the synthesis of shorter transcripts and stimulates the formation of longer ones (lanes 4–6). 30 min pre-incubation (either with or without factor Xa) has essentially no effect on the length of the resulting transcripts (lanes 7–9 and 10–12); the attached polymerase elongates just as efficiently as the free enzyme. Washing after preincubation with factor Xa removes >80 per cent activity (lanes 16–18; note that band intensities are weaker), showing that treatment with Xa detaches the polymerase. (From Cook and Gove 1992.)

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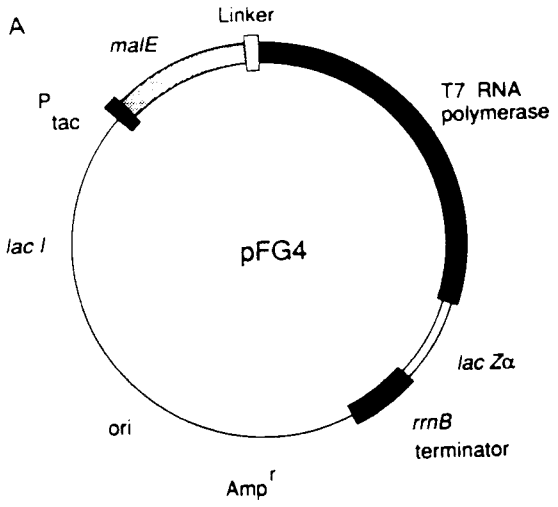

transcript; instead DNA rotates. Polymerase translocation along DNA generates positive supercoiling 'waves' ahead of, and negative supercoiling 'waves' behind, the moving enzyme. The torsional strain associated with these supercoils limits transcription unless removed by topoisomerases. Although there is now considerable support for such twin domains (e.g. Wu *et al.* 1988; Droge and Nordheim 1991), this model faces the problem of preventing the polymerase from rotating whilst allowing it to translocate. Even one accidental rotation—which is especially likely in long transcription units or when the transcript is short and frictional drag limited—would yield an entwined transcript. Heggeler-Bordier *et al.* (1992) have recently suggested that rotation might be restricted if the polymerase deformed the template into an apical loop, so preventing rotation of the loop and associated enzyme about the helical axis. But again, it seems unlikely that this could completely prevent rotation throughout long transcription units. Indeed, it is difficult to imagine any mechanism that would do so without immobilizing the polymerase.

In model 4, threading and untwining problems are completely eliminated because the enzyme is immobilized by attachment to some larger structure (i.e. the nucleoskeleton); instead DNA both translocates and rotates (Jackson *et al.* 1981; Cook 1989). It can be viewed as a special case of the 'twin-domain' model: domains of supercoiling are generated in much the same way and must be removed.

Are immobile polymerases active?

But can an attached polymerase work? Therefore we tested whether immobilization inhibited the activity of one of the most active polymerases known, that of the bacteriophage T7 (Fig. 6.8; Cook and Gove 1992). A bipartite protein consisting of the polymerase connected through a peptide linker with an immobilizing domain was expressed in bacteria. This was attached (via an antibody to the immobilizing domain) to protein A, which was, in turn, covalently linked to plastic beads. The polymerase could be released by cleaving the linker with a specific protease, factor Xa (Fig. 6.8 (*c*)). Comparison of the bound and free forms (i.e. after treatment without or with factor Xa) showed that immobilization reduced the rate of initiation but had little effect on elongation (Fig. 6.8(D)).

Fig. 6.9. A model for transcription. (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; they are probably adjacent transcription units. A gene out in the loop cannot be transcribed as its promoter (P) is remote from any attached polymerase. (E) marks an upstream activating sequence (e.g. an enhancer). (B) During development, the gene in the loop becomes active by attachment to a transcription complex assembled on the skeleton. The complex contains a polymerase (pol) flanked by two topoisomerases (T), plus a transporter (engine) on a track that leads through 'stations' where the appropriate enzymes for RNA processing, including polyadenylation (p(A)) and splicing (Sp) are concentrated. Initially E attaches at one site (triangle) to become permanently tethered to the complex; this inevitably brings P into close proximity to the polymerase, facilitating its binding. Elements of the complex are drawn spatially separated but they are probably in close contact to allow

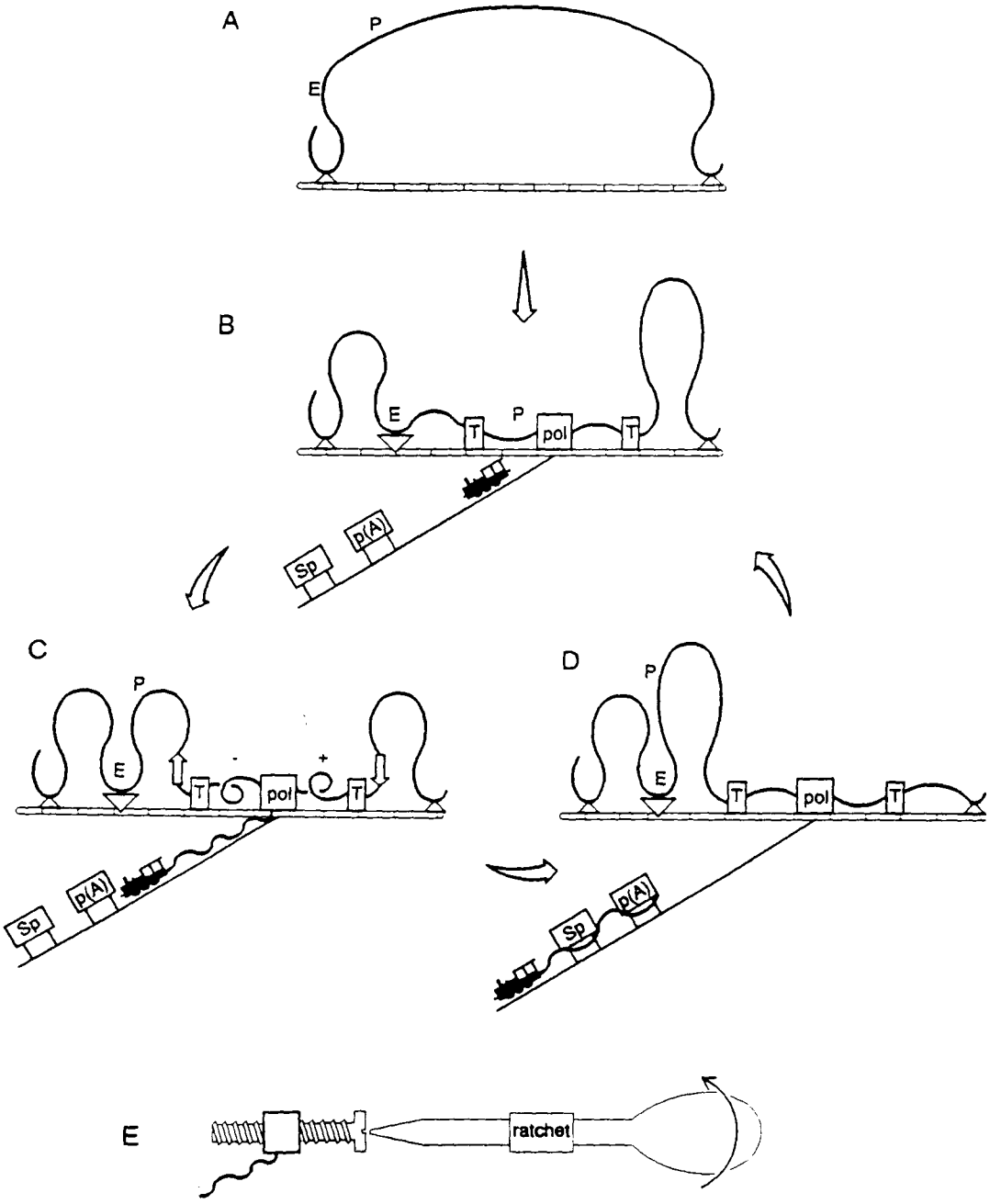

Fig. 6.9 *(cont'd)*

inter-communication. (C) After initiation, DNA moves (arrows) through the complex as RNA (wavy line) is extruded and attached to the transporter, which has begun to move down the track. The loop on the right shrinks as the loop on the left enlarges. Positive and negative supercoils appear transiently as shown but are removed immediately by topoisomerases. (D) The transcript is complete; it has been spliced and polyadenylated and is being transported to the nuclear pore. The template now detaches from the polymerase and the topoisomerases, but is held at the enhancer so that the promoter can easily rebind to start the whole process again. (E) Transcription is analogues to driving a bolt (DNA) through a nut (polymerase), whilst the ratchet (topoisomerase) in the screwdriver releases torsional strain. The complex is shown below the active transcription unit in (C). Adapted from Cook (1989).

Model for transcription

This leads us to a general model for transcription in which unentangled transcripts can only be made by immobilized enzymes. Bacteria and viral enzymes probably function as dimers, anchoring themselves to one piece of DNA whilst transcribing another, as in bacterial nucleoids. Eukaryotic enzymes adopt a different strategy, becoming immobilized by attachment to a skeleton (Fig. 6.9; Cook 1989). In the special case of the reactivating chick erythrocyte nucleus in the heterokaryon, the initially inert nucleus lacks a skeleton and its associated polymerases; chick genes are remote from polymerases on the mouse skeleton and cannot be transcribed. Only when a skeleton (plus associated transcription machinery) are built in the *chick* nucleus can promoters attach and the template move through the fixed polymerizing site to generate the transcript. Subsequent processing and transport also take place on the skeleton. Nuclear swelling and the appearance of nucleoli are then gross structural correlates of this complicated process.

The skeleton and replication

This essay has concentrated on the role of a nucleoskeleton during transcription. An integrating role for a similar structure during replication is also emerging (reviewed by Cook 1991) and what relationship there is between the two skeletons is obviously of the greatest interest.

Conclusions

These experiments lead us to a very different view of how transcription occurs—the DNA moves rather than the polymerase. People often say that movement is relative, so why should it matter which moves past the other? There are at least two very good reasons. First, I think it important to get the principles governing such a basic process as transcription right. It does not matter to most of us whether or not the earth goes round the sun, but we do like to know which moves. The second reason is more practical. Biochemists find it relatively easy to work with soluble enzymes found in supernatants, discarding pelleted material. But I think more authentic activities can be found in the pellet; we should concentrate on these, instead of throwing them away!

Acknowledgements

The Cancer Research Campaign has continuously supported me and my group over the years; recent work has also been supported by the Wellcome Trust and the Medical Research Council. I have summarized the work of many people, but Dean Jackson's contribution should be specially acknowledged. Henry Harris introduced me to 'many hours of simple pleasure' and has encouraged me throughout my career; I thank him for his help, especially when things were not going well.

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