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The Role of the Nucleoskeleton in RNA Metabolism

P.R. Cook, A. Dolle, A.B. Hassan, P. Hozak, and D.A. Jackson[1]

1 Introduction

The level of expression of the rat growth hormone gene in liver tumour cells is at least eight orders of magnitude less than that in anterior pituitary cells (Ivarie et al. 1983). This is to be compared with a 100-fold reduction in expression obtained by deleting promoters, enhancers or transcription-factor binding sites in plasmids prior to their transfection into tissue culture cells (Muller et al. 1988). Clearly, other important mechanisms must be superimposed upon the ones involving transcription factors that we know so much about. This essay will explore recent evidence that points to higher-order gene structure as a key determinant of activity.

2 Position Effects

The classical demonstration of position effect first indicated that a gene's activity critically depends on its particular location on a chromosome (Baker 1968). When active euchromatic genes in a fly are relocated close to a break in heterochromatin, they assume the genetic inactivity that characterizes the heterochromatin. Such repressive effects are remarkable in that *every* gene seems susceptible to them, with the repression able to spread along the chromosome from the heterochromatic breakpoint. Overriding effects of position on expression have also been seen recently in transgenic mice. Insertion of a reporter gene (e.g. β-galactosidase) into different chromosomal sites in different animals leads to very different levels of expression (e.g. Allen et al. 1988); activity depends critically on the precise "chromosomal context" into which the transgene is inserted. Inserted reporter genes can be insulated from repression by adjacent chromatin by sequences flanking the β-globin and *Drosophila* hsp70 loci (e.g. Grosveld et al. 1987; Kellum and Schedl 1991). These observations point to some gross structural mechanism that profoundly influences gene activity.

[1] Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford, OX1 3RE, UK

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3 Spatial Organization of Post-Transcriptional Processing

Our views on how the pathways involved in processing nascent RNA are organized have recently changed, largely as a result of the introduction of the techniques of high-resolution immunofluorescence. Traditional views were based upon the unstated assumption that the enzymes involved were soluble and freely diffusible: RNA polymerases diffused to the promoter and, after initiation, would then process along the DNA; next, the nascent RNA would be processed (e.g. capped, spliced, methylated, polyadenylated) by soluble enzymes that might come together briefly in complexes before disassembling to reform again on different transcripts; finally, the processed transcript would diffuse through the nucleoplasm to the nuclear pores and thence to the cytoplasm. According to this view, sites of synthesis and processing would be expected to be spread throughout the nucleus. However, recent evidence points to a highly structured processing pathway, during which nascent transcripts are associated throughout with some solid phase in the nucleus.

Initial evidence for some higher level of organization came from immunolocalization studies using serum from patients suffering autoimmune disease. Some of the sera – often those which selectively precipitated small nuclear ribonucleoprotein particles involved in pre-mRNA splicing (e.g. Sm antisera) – recognized antigens concentrated in "speckles", rather than spread throughout the nucleus (reviewed by Carter et al. 1991). Antisense probes against snRNPs that are integral parts of the spliceosome reveal similar speckles (Carmo-Fonseca et al. 1991). Specific sequences seem to be responsible for targeting proteins to these areas (Li and Bingham 1991) which are also rich in a spliceosome-assembly factor, nascent RNA and poly(A) (Fu and Maniatis 1990; Carter et al. 1991; Huang and Spector 1991). Furthermore, it is intriguing that both vaccinia and influenza viruses encode single polypeptides with two enzymic activities (Beaton and Krug 1986; Shuman et al. 1987; Schierle et al. 1992), one that works at the 5′ end (i.e. a capping or methylation activity) and the other at the 3′ end of mRNA (i.e. during termination or polyadenylation), implying that the two activities must be co-localized. All these results are consistent with splicing and polyadenylation occurring in local regions where the relevant activities are concentrated. However, and this is an important *caveat*, they could equally reflect sites of storage and so do not provide decisive evidence for focal sites of *activity*.

Perhaps the most striking result consistent with a role for some underlying stucture comes from the work of Lawrence and coworkers (Lawrence et al. 1989; Xing and Lawrence 1991). They localized transcripts within swollen nuclei from cells latently infected with Epstein Barr virus; the virus had integrated at two closely spaced sites within the host genome. In situ hybridization using biotinylated probes and fluorescently labelled avidin did not reveal the diffuse pattern expected of nascent RNA that had diffused

throughout the nucleus, but curvilinear "tracks" extending from the internal genes towards the periphery. This pattern remained even after much of the chromatin had been removed and implies that transcripts are transported along some skeleton from gene to nuclear membrane. But again, such results would be obtained if the message was unattached to any structure and diffused through channels in the dense chromatin. If the diffusible message aggregated whilst most chromatin was being removed, then such "tracks" would resist extaction and suggest that the message was attached to some underlying structure.

Further evidence consistent with a role for some underlying transport network comes from the analysis of signals governing the export of nascent RNA (reviewed by Hamm and Mattaj 1990). U1 snRNAs are normally made by RNA polymerase II and are exported to the cytoplasm. However, if the U1 gene is put under the control of an RNA polymerase III promoter, the resulting transcripts remain in the nucleus. The ultimate destination of the RNA is clearly influenced by the type of polymerase used for its synthesis. At least two other major factors influence export of RNA molecules – the structure of their 5′ caps and whether they contain introns: monomethylation of caps and splicing both facilitate export. A complicated model based on the order of capping and splicing has been proposed to explain how transcripts are directed to the appropriate destinations (Hamm and Mattaj 1990). Although traffic control could be exercised by soluble enzymes acting successively on freely diffusible transcripts, the polymerase could equally deposit a transcript onto a particular track which inevitably took it through processing "stations" to its destination.

All these results suggest, but certainly do not prove, that the machinery involved in post-transcriptional processing is spatially well organized, perhaps at the junctions on a network along which the transcripts pass. How might the RNA polymerase be connected to this network?

4 Attached Polymerases

Biochemical evidence has long shown that most RNA polymerase II in the cell is associated with insoluble nuclear material (Beebee 1979; Weil et al. 1979; Jackson and Cook 1985). Furthermore, it is not widely appreciated that the soluble activity isolated by most biochemists is transcriptionally inefficient and only becomes active when incorporated into large complexes. For example, 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 apparently "soluble" activities in extracts quickly form very large complexes.

However, transcription complexes are very sticky, and so it is inevitable that there is the suspicion that such aggregates are generated artefactually.

The same is true of the many observations showing that nascent RNA is associated with nucleoid "cages" or nuclear "matrices", which are isolated using high concentrations of salt (Cook 1988). Such unphysiological conditions are used because chromatin aggregates into an unworkable mess at isotonic salt concentrations. More physiological conditions can be used if cells are encapsulated before lysis in agarose microbeads of about 50 μm diameter. 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 (Jackson et al. 1988). These fragile cell remnants are protected by the agarose coat, but are accessible to probes like antibodies and enzymes. Whilst one cannot be certain that any isolated is artefact-free, this contains intact DNA and essentially *all* the replicative and transcriptional activities of the living cell. Most chromatin can be removed by a combined nucleolytic and electrophoretic treatment (all in a "physiological" buffer) without reducing these activities. If artefacts are generated, they cannot interfere with these vital functions.

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. (Note that chromatin-containing DNA fragments of 150-kb DNA can escape from beads.) Cutting HeLa chromatin into 10-kb fragments or less, followed by electroelution of most of the chromatin, leaves residual clumps of chromatin associated with an intermediate filament-like skeleton (Jackson and Cook 1988). However, removal of the chromatin hardly reduces the activity of RNA polymerases I and II (Jackson and Cook 1985; Dickinson et al. 1990) or DNA polymerase α (Jackson and Cook 1986; Jackson et al. 1988). Nascent RNA, nascent DNA and transcribed templates, even as minichromosomes only a few kilobases long, also resisted electroelution (Jackson and Cook 1985; Jackson and Cook, submitted).

The use of "physiological" conditions and recovery of essentially *all* activity, rather than a minor fraction, make explanations based on artefacts involving aggregated polymerases difficult to sustain. These results are simply explained if polymerases are attached. 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.

5 The Topology of Transcription

Transcription of a double helix poses various topological problems. One concerns templates with ends that are unable to rotate freely, for example

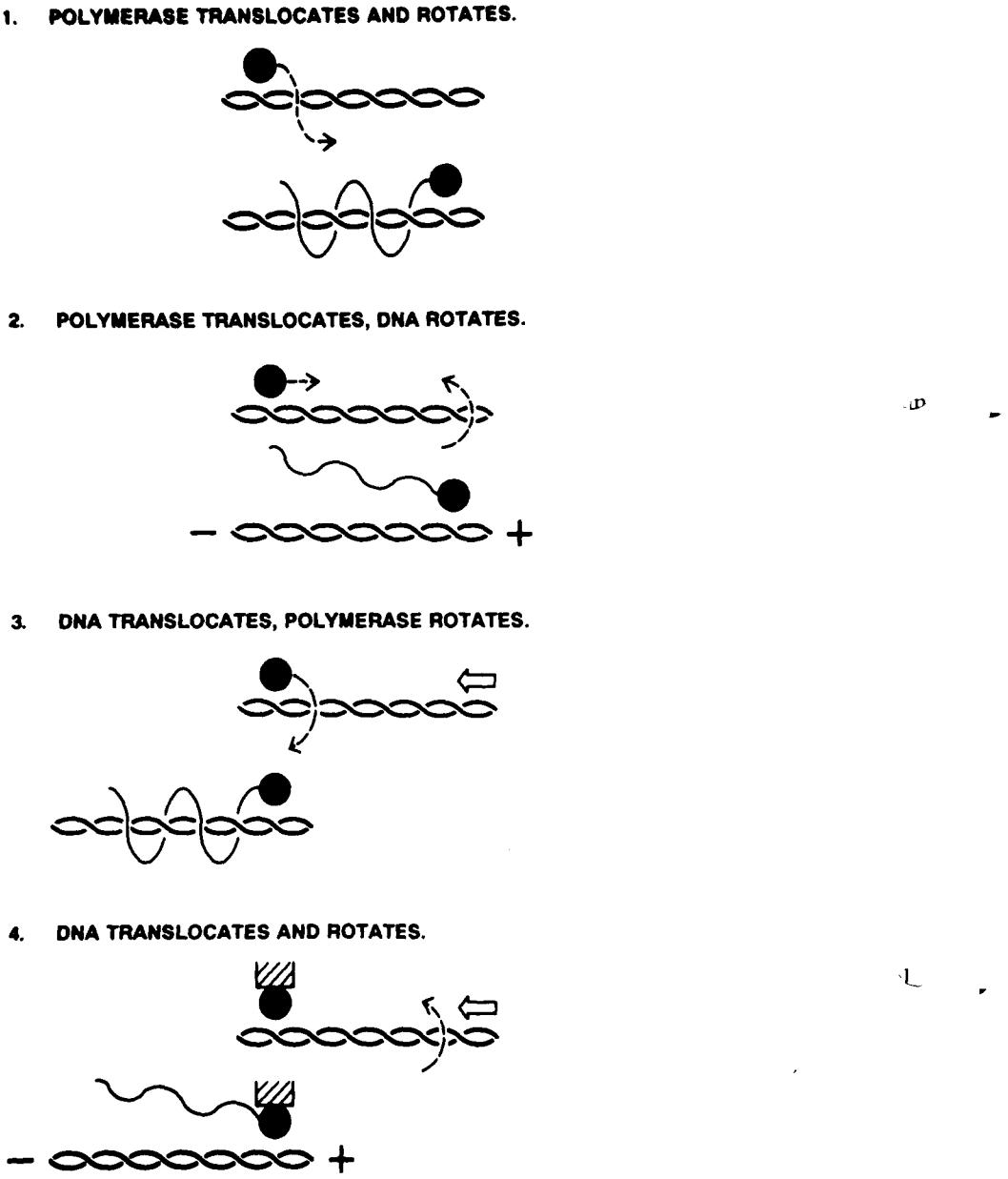


Fig. 1. Models for transcriptional elongation involving mobile or static polymerases (*black circles*) and double-helical templates. *Upper panels* in each model indicate initial relative positions; *arrows* show subsequent movements. *Lower panels* illustrate final positions after transcription. + and − indicate domains of positive and negative supercoiling. In **4**, the *hatched area* immobilizes the polymerase and the resulting transcript is not entwined about the template (Cook and Gove 1992)

those organized into circles or loops (Liu and Wang 1987). Another concerns the interlocking of template and transcript that results if the polymerase tracks along a helical strand, as in the models found in most textbooks. 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 player (polymerase or DNA) performs which movement (rotation or translocation).

The first model in Fig. 1 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 base pairs 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 both models 1 and 3 unlikely.

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 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 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; 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. Active polymerases would be immobilized if they anchored themselves to one piece of DNA whilst transcribing another, as in bacterial "nucleoids", or if they were attached to some skeleton.

6 Are Immobile Polymerases Active?

We are so used to thinking that polymerases are soluble enzymes that it becomes important to demonstrate that attached polymerases are indeed

active. Two recent studies have done so. Schafer et al. (1991) immobilized the RNA polymerase of *E. coli* on a glass slide and added to it a template, with a promoter at one end and a gold particle at the other. They saw two kinds of particles in the light microscope, one moving with Brownian motion in three dimensions, the other restricted to a small volume about a point on the slide. Presumably, some templates were free whilst others had become tethered to the enzyme on the slide by promoter-binding. When transcription was initiated, the movement of the tethered particles became even more restricted as the template moved past the fixed polymerase and the length of the tether decreased. The rate of elongation was deduced from the rate at which the tether decreased; it was similar to the rate found with the soluble enzyme.

The second study involved the direct immobilization of a polymerase (Cook and Gove 1992). A bipartite protein consisting of the RNA polymerase of T7 bacteriophage, 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. Polymerase could be released by cleaving the linker with a protease, factor Xa. Comparison of the activity of the bound and free enzymes showed that immobilization reduced the rate of initiation but had little effect on the elongation rate.

These experiments show that immobilization is no barrier to elongation, at least with these simple enzymes.

7 Nucleoskeletons

These observations obviously beg the question: to what might polymerases be attached? Unfortunately, there is little agreement as to the nature of any nucleoskeleton. Therefore, it is as well to begin any discussion with some disclaimers. First, the various synonyms of nucleoskeleton (e.g. matrix, scaffold, cage) imply stability, but the true skeleton is probably disassembled and then reassembled. Second, we talk of one skeleton: there may be many, some related (e.g. mitotic and interphase skeletons) and others unrelated, both structurally and functionally, like the different cytoskeletal elements. Third, there are methodological problems in visualizing a nucleoskeleton. For example, the immunofluorescence pictures described above almost always show "speckles" mentioned earlier and not a filamentous network analogous to the cytoskeleton. But the skeleton might be too diffuse to be detected in this way, with epitopes too weakly immunogenic or inaccessibly buried in chromatin. However, the main reason why the nucleoskeleton remains so elusive and controversial is because candidate structures (e.g. matrices, scaffolds, cages) are isolated using such markedly unphysiological conditions that they may simply be isolation artefacts, with no counterparts in vivo (Cook 1988).

Ultimately, the controversy can only be resolved by studying structure in vivo or using physiological conditions. The intermediate filament-like skeleton described above was seen using a "physiological" buffer and so is a strong candidate as the skeleton to which the various components of the transcriptional pathway are associated, and we are currently investigating whether this is so. In this context it is interesting to note that several transcription factors, including the products of the *fos*, *jun*, *creb* and *TPR* genes, are related to intermediate filaments (which are formed by aggregation of subunits) and so might well complex with such a skeleton (Capetanaki et al. 1990). However, until the skeleton is defined in molecular detail, its existence will remain controversial. The history of the nucleoskeleton may be repeating that of the cytoskeleton; its existence was disputed until antibodies directed against pure components were obtained and found to decorate the various cytoskeletal systems.

8 A Model for Transcription

Our intuition that polymerases track along DNA stems from a perception of relative size – the smaller of the two moves. However, if polymerases are attached to some skeleton and the template subdivided into chromatin domains of a few tens of kilobases, the template becomes smaller than the enzyme complex; then the DNA in one loop could move through an attached polymerizing site (Figs. 2 and 3). As chromatin is relatively inflexible, some regions of the template can *never* approach the polymerization site and, being out in a loop, would never be transcribed. 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 genes within range and they would become active only be attachment. The dedication of polymerases to transcribe particular genes is the inevitable consequence of this model. It is easy to imagine how stable transcription complexes might be formed and how specific attachments – and so specific patterns of expression – might be inherited through cell division (Cook 1989). Note that all ends of nascent RNA are attached so that they cannot become entangled.

This model suggests that nascent RNA spends its entire life within the nucleus associated with the same structure, from synthesis to export (Jackson et al. 1981; Cook 1989). Presumably, the message is actively transported along nucleofilaments to the nuclear pores, perhaps where it becomes associated with ribosomes and transhiped to a different transporter running along a different set of tracks (e.g. the cytoplasmic actin network). This model allows specific targeting of messages to specific cellular locations and a flow of information in reverse from membrane to gene. Contacts between cell membranes generated during differentiation might stabilize underlying cytoskeletons, and hence nucleoskeletons, which in turn could influence

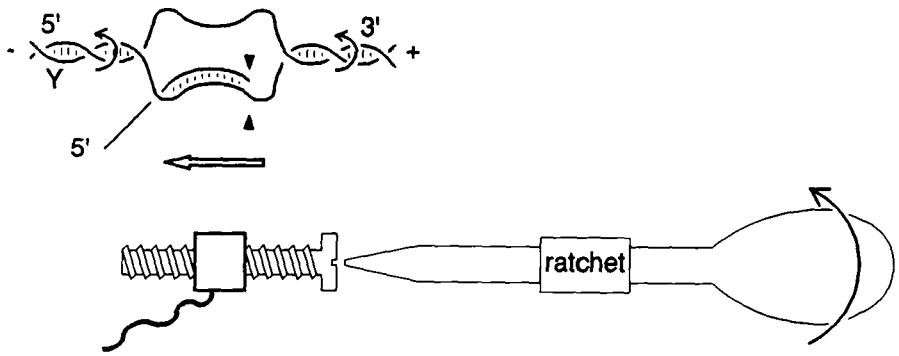


Fig. 2. A micromodel for transcription showing DNA movements at the polymerization site. DNA moves through the fixed polymerization site (*above*) like a screw through a fixed nut (*below*).

Above Y was the first base to be copied. DNA moves to the left (*arrow*) and spins (*arrows*) so that the transcribed base between the *triangles* always retains the same stereochemical relationship to the page (i.e. the skeleton). RNA is synthesized and extruded downwards to the left. Rotation induces compensatory supercoils (+ and −) to accumulate.

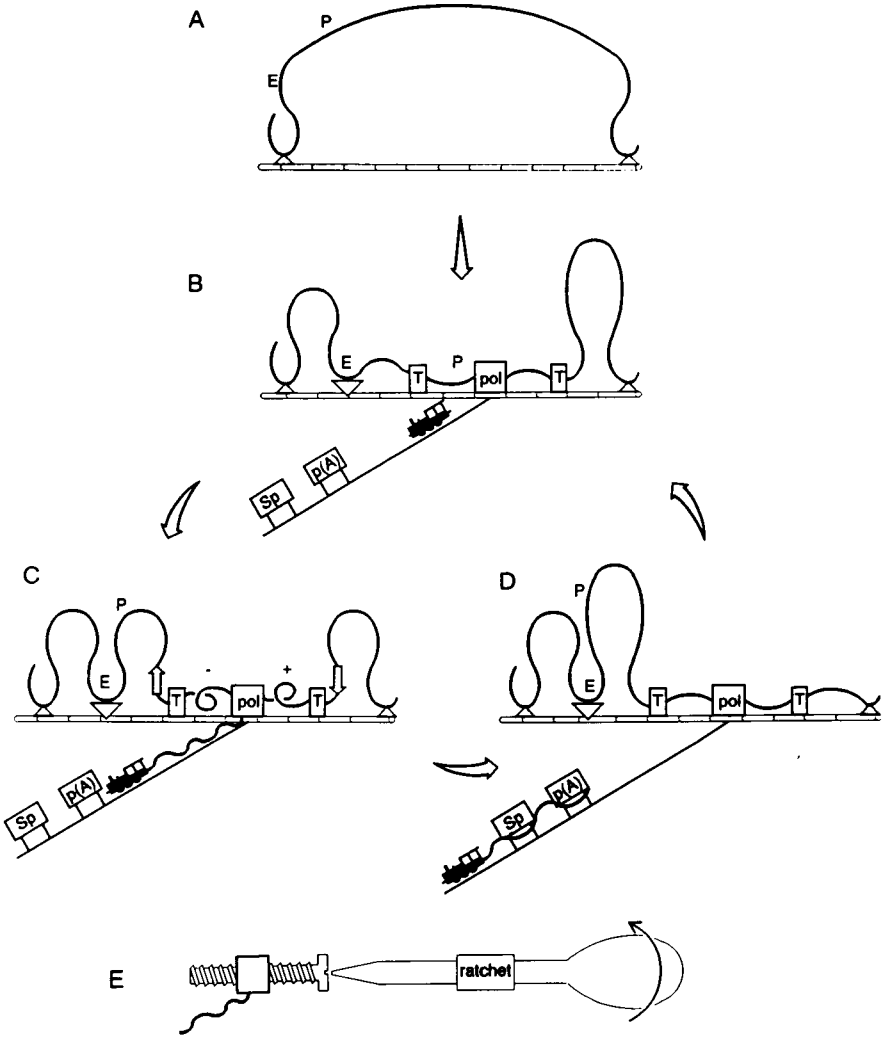
Below Template movements are analogous to those of a bolt (DNA) driven though a fixed nut (polymerase) using a ratchet (topoisomerase) screwdriver. The nut "sees" the whole length of the thread as it passes through; the fixed polymerase "sees" the transcribed strand in the same way. As a right-handed 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. Highly active transcription units would contain additional polymerases (nuts) between topoisomerases. (After Cook 1989)

Fig. 3A–E. A macromodel 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 may be 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 the appropriate 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 intercommunication. **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** The analogous nut and bolt are shown below the active transcription unit in **C**. (After Cook 1989)

gene expression. In this case it is the structure, rather than a second messenger, that transmits the information.

9 Higher-Order Structure 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). What relationship there is between the two skeletons is obviously of the greatest interest.



10 Conclusions

Current biochemical techniques are ill-adapted to the study of macromolecular complexes, especially those containing native templates which are so fragile; on disruption they become viscous and highly charged, aggregating easily. So inevitably, a first step during isolation involves breaking the complex into more manageable pieces, or extraction under unphysiological conditions. Then it is not surprising that *soluble* polymerizing activities are recovered. But it is rarely appreciated what a small fraction of the polymerase is recovered in a soluble form and how *inefficient* that minor fraction is. Most of the RNA polymerizing activity is associated with some insoluble nuclear fraction. Moreover, several lines of evidence now suggest that various post-transcriptional activities (e.g. splicing, polyadenylation, capping, transport) are attached to a larger organizing structure. Then the enzymes involved in the different steps of RNA metabolism are all associated with a solid phase in the nucleus. Consequently, we should not study the supernatant and discard the pellet, as many biochemists have done in the past; rather we should concentrate on the activities in the pellet.

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The Mechanism of Action of the Retinoblastoma Gene Product

N.B. La Thangue[1]

1 Introduction

It is has now been over 20 years since Knudson proposed that mutation in the retinoblastoma (Rb) gene is the genetic basis of the rare recessive childhood cancer, retinoblastoma (Knudson 1971). Since then, the Rb gene product (pRb) has established itself as a protein of central importance to those researchers interested in the regulation of cellular proliferation and the mechanisms of cellular transformation. Its biological properties do, however, differ in one important respect from the dominant growth-promoting effects of the ever expanding plethora of proto-oncogenes because it affects proliferation negatively, rather than positively. Thus, pRb has been labelled a tumour suppressor or anti-oncogene. Accordingly, the Rb gene is frequently mutated in tumour cells isolated from a variety of sources and pRb is sequestered by certain viral oncoproteins, effects that are assumed to inactivate its growth-regulating properties. Despite a wealth of information, however, its mechanism of action has remained an enigma. The purpose of this review is to collate recent developments in the field that now suggest a mechanism for how pRb exerts the biological effects of negative growth control.

2 The Retinoblastoma Gene Product Is Cell Cycle-Regulated and Sequestered by Viral Oncoproteins

The Rb gene, which is located on chromosome 13, encodes a 105-kDa nuclear protein constitutively expressed in many cell types of the adult mammal. It is widely believed that phosphorylation plays an important role in regulating pRb because wild-type pRb is phosphorylated in a cell cycle-dependent fashion, being hypophosphorylated in early G1 and undergoing a series of further phosphorylation events as the cell cycle progresses to reach a maximum level at the G2/M transition (De Caprio et al. 1992). Furthermore, since introduction of wild-type pRb into cells that carry a mutated allele arrests cell division in G1 (Goodrich et al. 1991), it is thought

[1] Laboratory of Eukaryotic Molecular Genetics, MRC National Institute for Medical Research, Mill Hill, London, NW7 1AA, UK

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