RNA Polymerase: Structural Determinant of the Chromatin Loop and the Chromosome

Peter R. Cook

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Summary

Current models for RNA synthesis involve an RNA polymerase that tracks along a static template. However, research on chromatin loops suggests that the template slides past a stationary polymerase; individual polymerases tie the chromatin fibre into loops and clusters of polymerases determine the basic structure of the interphase and metaphase chromosome. RNA polymerase is then both a player and a manager of the chromosome loop.

Introduction

The current model for RNA synthesis involves a polymerizing complex that tracks along the template – the enzyme moves whilst the DNA remains stationary (Fig. 1A); if such is the case, it is unlikely that the moving enzyme could form the skeleton that determines chromosome structure. An alternative model sees the active polymerase attached to a large structure in the eukaryotic nucleus with RNA being made as the template slides through a fixed polymerization site[1] (Fig. 1B); in this view, the enzyme becomes a major determinant of chromosome structure. The term 'polymerase' is used here to describe the cluster of many different polypeptides that form the active complex[2,3].

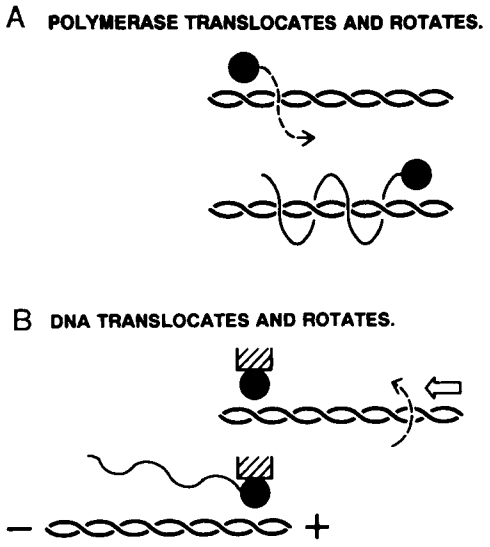

Fig. 1. Models for transcription. (A) The polymerase (solid circle) tracks (arrow) along one of the two helical strands to generate a transcript that is entwined about the static template. (B) DNA rotates (arrow) as it slides (large arrow) past the immobile polymerase (solid circle attached to hatched area); the transcript is extruded as template rotation generates positive and negative supercoils (indicated by + and –) which must be removed by a topoisomerase. The movements involved are similar to driving a screw (DNA) through a fixed bolt (nut). (Redrawn from ref. 9.)

Mobile polymerases

The idea that RNA polymerases track along the template pervades our thinking. It stems from a perception of relative size and the preconception that it should be the smallest object – the polymerase – that moves. Indeed, RNA polymerases can be photographed apparently tracking along the template[4]. However, these beautiful images of 'Miller' spreads are obtained by osmotically disrupting cells to spread the dense chromatin and then photographing the edge of the spread; most polymerases, in fact, remain in the central mass and the few photogenic ones at the periphery might have been stripped away from a larger structure during spreading.

Other evidence is indirect but compelling; the argument runs as follows. Pure soluble polymerases transcribe pure templates *in vitro*, so why invoke any role for a larger structure *in vivo*? However, an immobile activity *in vivo* might become a mobile and less active one *in vitro*. It is not often appreciated how inefficiently pure polymerases initiate on intact chromatin templates and although crude preparations initiate correctly (but inefficiently), they do so after long preincubations when activities assemble into complexes large enough to be pelleted by a short spin in a microcentrifuge[5]. Moreover, transcription factors and enhancers stimulate such reactions by a few hundred-fold or less[6], whilst *in vivo* the transcription rate of the growth hormone gene probably varies a hundred million-fold during development[7]. Clearly, something important is missing from such *in vitro* reactions.

Immobile polymerases

But can immobile polymerases work? Two experiments show they can. When the RNA polymerase of *E. coli* is immobilized on a glass slide and mixed with a template that has a promoter at one end and a gold particle at the other, two kinds of particles are seen in the light microscope[8]. One moves with Brownian motion, the other is restricted to a small volume about a point on the slide; presumably some templates are free whilst others are tethered through the promoter to the bound enzyme. When transcription is initiated, the tethered particles become even more restricted in their movement as the template slides past the attached polymerase and the length of the tether decreases. The elongation rate – deduced from the rate at which the tether decreases – is similar to that found with the soluble enzyme.

The second study involved attaching a modified bacteriophage polymerase to plastic beads; the intact polymerase could be released by protease-cleavage[9]. Although the bound and free enzymes initiate at different rates, they again elongate similarly. Clearly, these immobilized enzymes work.

Topological considerations also suggest that polymerases must be immobile. A tracking polymerase rotates once about the template every ten base-pairs, so the transcript becomes entwined about the template (Fig. 1A). To my knowledge, no practical mechanism for untwining the two has yet been suggested. As some transcription units are tens of thousands of base-pairs long, their transcripts will be entwined thousands of times. Even if frictional drag in the transcript restricts rotation of the polymerase[10], one accidental rotation will still entangle the transcript so it cannot get out to the cytoplasm. This untwining problem simply does not arise if DNA rotates as it moves past an immobile enzyme (Fig. 1B).

Any movement is, of course, relative. This means that although a template may move relative to a polymerase, both could move together relative to an external viewpoint (e.g. as chromatin domains tumble during interphase or as chromosomes segregate during mitosis).

Artifacts

Physiological conditions are rarely used during polymerase assay, or when isolating chromatin. Chromatin is probably designed so that small alterations in its charge change its structure, triggering concomitant changes in function. This has the unfortunate corollary that it aggregates into an unworkable mess in isotonic saline. Therefore biochemists use more tractable conditions and often isolate it in (at least) one-tenth the physiological salt concentration. But this destroys the 30 nm chromatin fibre, extracts a quarter of nuclear protein, aggregates ribonucleoprotein particles and generates a new attachment of the chromatin fibre to the substructure for every one that pre-existed. Often residual aggregation is suppressed by adding 'stabilizing' cations, but these generate further artifactual attachments.

The use of such unphysiological conditions causes much of the controversy concerning the molecular basis of chromosome structure[11]. The different conditions used to isolate sub-nuclear structures like 'matrices', 'scaffolds' and 'cages' ensure that each has its own characteristic set of sequences associated with a different sub-set of proteins. For example, **m**atrix-**a**ttached **r**egions or 'MARs' are bound to various different proteins, depending on the precise method of isolation; **s**caffold-**a**ttached **r**egions or 'SARs' are often specifically associated with topoisomerase II, and transcribed sequences are bound to cages. Sceptics suggest that these various complexes are all artifacts and have no counterparts *in vivo*. After reviewing the initial evidence for fixed polymerases which relied on the use of hypertonic conditions, I will concentrate on results obtained using more physiological conditions.

Nucleoids

Lysing bacterial spheroplasts in 1 M NaCl releases the circular genome, which is folded through its association with engaged RNA polymerases into discrete supercoiled domains[12,13]. Lysing eukaryotic cells releases analogous 'nucleoids' consisting of a residual nuclear skeleton or 'cage' associated with loops of superhelical DNA[14]. Despite the bacterial precedent, it originally seemed unlikely that an RNA polymerase could organize the loops; the concept of a tracking skeleton was just too bizarre! It was more natural to assume that sequences at the base of each loop were attached to a structural protein in the cage, but despite many attempts at molecular characterization there is still no consensus as to what those sequences and proteins might be.

Several observations prompted us to examine whether polymerases might fold DNA into loops[15]. First, whilst human nucleoids contained no polymerizing activity, they did retain all nascent RNA, implying that the transcripts were held by an inactive enzyme. Second, polymerizing activity and attachments to the cage were lost concurrently as chick erythroblasts matured into inert erythrocytes. Third, electron microscopy showed nascent transcripts attached to the cage and not to the body of the loop, implying that polymerases were at the cage. Fourth, cutting loops with *Eco*RI detached most DNA but left transcribed regions and enhancers; cutting with RNAase removed the middle of nascent transcripts but not the ends. These results are simply explained if transcription occurs as the template slides past polymerases fixed to the cage, generating transcripts attached at both ends[16].

Agarose beads and 'physiological' conditions

Although these attachments seen in nucleoids were specific, they could have arisen artifactually during isolation. Fortunately, problems caused by aggregation at an isotonic salt concentration can be sidestepped if cells are first encapsulated in agarose microbeads (25-150 μm diameter) and then lysed in a 'physiological' buffer. The protective coat of agarose prevents aggregation yet allows molecular probes (e.g. enzymes, antibodies) access to the template; the resulting chromatin retains its integrity (assayed by the presence of supercoiling after removing histones) and essentially all the replicational and transcriptional activity of the living cell. If attachments of polymerases to an underlying skeleton were generated artifactually, we would expect them to lose activity. A biochemist can never completely rebut the criticism that he generates an artifact when he breaks open a cell, but this approach – which uses conditions as close to the physiological as is conveniently possible and which preserves activity of the structure under study – is perhaps the best that can be done, short of studying the living cell.

Models involving mobile or immobile (i.e. attached) polymerases can be distinguished by cutting the encapsulated chromatin with an endonuclease into fragments of <10 kb and then removing ~90% of the fragments by electrophoresis (Fig. 2). If polymerases track around the chromatin loops, then ~90% activity should elute with the fragments. In fact, most activity remained in beads, suggesting it was attached to some kind of skeleton[17].

After removing most chromatin, loop size can be deduced from the size of the residual attached fragments and the percentage of chromatin remaining in beads[18]. Sizes ranged from 5-200 kb (average = 86 kb); the smaller loops are probably transcriptionally active. Loops in nucleoids were slightly larger, suggesting that some attachments had been disrupted. Loops in nuclei isolated by conventional methods, as well as matrices and scaffolds – which all spend some time

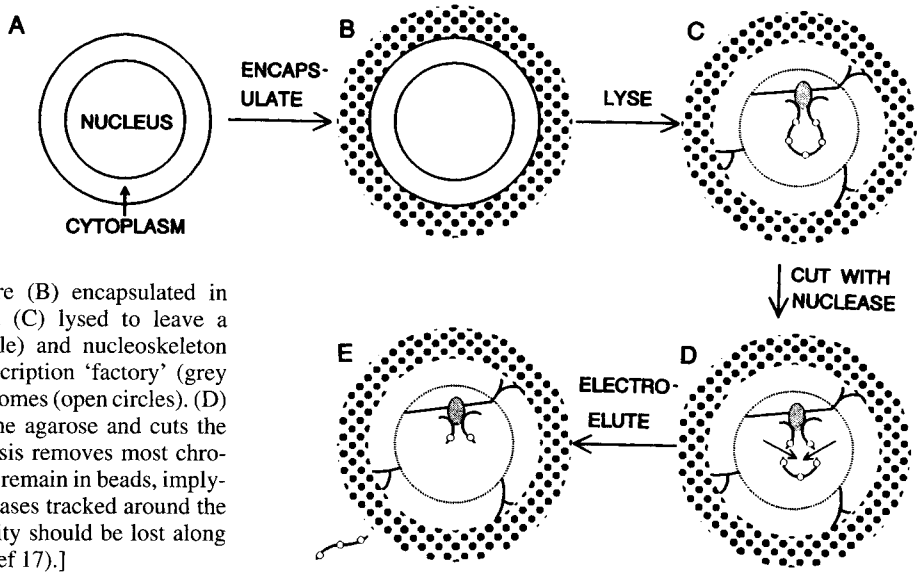

Fig. 2. Do polymerases track around chromatin loops or are they attached to an underlying structure? (A) Cells are (B) encapsulated in agarose beads (dotted surroundings) and (C) lysed to leave a cytoskeleton, nuclear lamina (dotted circle) and nucleoskeleton (straight line) to which is attached a transcription 'factory' (grey oval) and a DNA loop covered with nucleosomes (open circles). (D) An added endonuclease difuses through the agarose and cuts the chromatin loop (arrows). (E) Electrophoresis removes most chromatin. All polymerizing activity is found to remain in beads, implying that the enzyme is attached. If polymerases tracked around the loop (not shown), then polymerizing activity should be lost along with the eluted chromatin. [Redrawn from ref 17).]

in hypotonic buffers – had smaller loops, which must have been generated during isolation.

Transcription foci and factories

Seeing is believing. The idea that post-transcriptional events like splicing and transport take place on a solid phase in nuclei has been boosted by the visualization of immunofluorescent 'speckles' and 'tracks', where splicing components and completed transcripts are concentrated[19,20]. (But note that the 'tracks' could reflect precipitates of mRNA that originally diffused through channels in chromatin, rather than active transport along an underlying solid phase[21].) Recently the synthetic site has been tied into this solid-phase network[22,23]. Encapsulated and permeabilized HeLa cells were incubated with Br-UTP; then 300-500 fluorescent foci were immunolabelled using an antibody against Br-RNA (Fig. 3). The foci contain RNA polymerase II and Sm antigen, a component of the splicing apparatus. Calculations suggest that each focus contains ~50 active polymerases and many templates, so we call them transcription 'factories'. α-amanitin, an inhibitor of RNA polymerase II, prevents incorporation into these foci and then ~25 nucleolar foci become visible. Both nucleolar and extra-nucleolar foci remain after removing most chromatin, confirming that synthetic sites are attached to an underlying skeleton. We might have expected tracking polymerases to be spread throughout 'open' chromatin, but their concentration into a focus clearly shows that they do not have the freedom to track everywhere.

Model loops

Many polymerases could track around a cluster of loops and so resist elution in the experiment illustrated in Fig. 2, if loops were too small to be cut. However, experiments with a minichromosome confirm that active polymerases resist elution even when the chromatin fibre is shredded into very small pieces[24]. The minichromosome possessed the SV_{40}

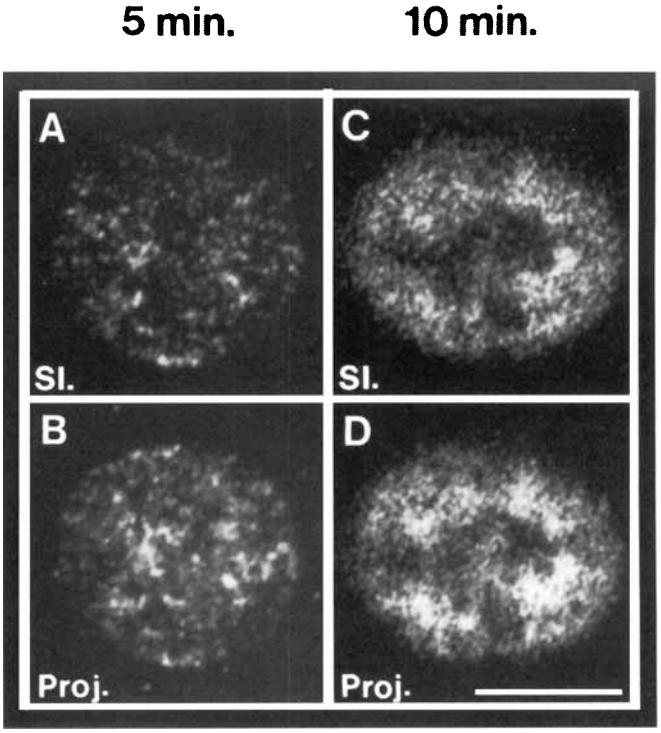

Fig. 3. Transcription sites visualized by 'confocal' microscopy. HeLa cells were permeabilized with streptolysin O, incubated with Br-UTP for 5 min (A,B) or 10 min (C,D) to extend nascent RNA chains by ~200 and ~400 nucleotides, respectively, and sites containing Br-RNA were indirectly immunolabelled. Nine optical slices were taken through a typical nucleus from each sample; A and C show a central slice (Sl) and B and D the projections (Proj) of the nine sections on to a single plane. Transcription sites are not spread throughout nuclei, but concentrated in foci or 'factories'. Bar, 5 μm. (From ref. 22, by permission of Oxford University Press.)

origin of replication plus transcription units under the control of the SV_{40}-early and human α1-globin promoters; it grows in cos7 cells, which express SV_{40} T antigen to give two populations – an inert fraction that eluted (i.e. was unattached) and a transcribed fraction that resisted elution. After cutting each attached minichromosome into ~400 bp fragments, ~90% of the contour length eluted; the remaining fragment or two were associated with a still-active polymerase or a promoter.

These results held several surprises. Active minichromosomes might have been attached through a common sequence like an origin or SAR, but no single sequence was uniquely responsible for attachment; rather, different parts of each minichromosome were attached at one or, at most, two points. Moreover, both transcription units are highly active in these cells and might have been packed with polymerases as in 'Miller' spreads of ribosomal genes, but there was only one per minichromosome, consistent with transcription of one gene 'interfering' with that of an adjacent gene[25].

This gives us a very dynamic view of loop structure. Inert minichromosomes attach to a factory to become active, initially at one of the two promoters. Then a polymerase engages and the minichromosome becomes attached solely through the sequence at the polymerizing site. Finally the template dissociates, but its proximity to binding sites in the factory will mean that it can compete effectively with other minichromosomes for those binding sites. Therefore, at any moment individual templates will be at different stages in this cycle; no one point is always attached and different points have different probabilities of attachment.

Nucleolar factories

The nucleolus provides us with a model for a transcription factory. Several 'fibrillar centres' – which equal the number of polymerase I foci or factories described above – are each surrounded by a 'dense fibrillar component' embedded, in turn, in a 'granular component'[26]. The fibrillar centre is probably a store, containing polymerase I, topoisomerase I and the transcription factor, UBF. The polymerase directly organizes the structure, since its formation in mammalian cells is prevented by microinjecting antibodies to the enzyme[27] and yeast mutants with a deleted gene for the second largest subunit of the polymerase assemble several 'mininucleolar bodies' rather than a normal crescent-shaped nucleolus[28]. We imagine that ribosomal cistrons slide through the dense fibrillar component on the surface of the fibrillar centre as nascent rRNA is extruded[29].

Polymerase II factories would also be constructed around an internal store with different templates sliding through polymerases on its surface; nascent transcripts would again be extruded through neighbouring processing sites.

Speculations on mitotic chromosome structure

Models of the interphase eukaryotic chromosome and the bacterial chromosome are then similar; DNA in both is segregated into discrete loops, tied together by active polymerases at the bases. Such ties are some of the most stable known and survive in 2 M NaCl. They – and attachments through promoters and enhancers – probably constitute the major class of attachments in higher cells. This does not exclude the possibility that there are other kinds, perhaps involving SARs and MARs; however, these have only been seen after using unphysiological conditions that generate artifactual attachments[18]. But do polymerases also define loop structure during mitosis?

Given the extent of chromatin condensation, it is perhaps surprising that the contour length of loops – whether measured in 2 M NaCl or in a 'physiological' buffer – remains unchanged through mitosis, implying that the ties persist[18]. And although nascent transcripts abort at mitosis[30], polymerases I and II both remain bound quantitatively[31] so they probably continue to act as ties.

Nucleolar factories also provide a paradigm for the structural re-organization of mitosis. As HEp-2 cells enter mitosis, the huge stores of the polymerase I transcription factor, UBF, remain bound at 6-8 of the 10 nucleolar organizing regions (NORs). Subsequently these local concentrations are symmetrically partitioned amongst daughter cells[32]. Here the remnants of the interphase factory – the fibrillar centre and its surroundings – persist into metaphase as visible entities, the NORs. Perhaps polymerase II and III factories collapse on to each other to form the axial chromomeres of prophase, and subsequent condensation of differently sized loops on to those factories generates the bands typical of metaphase[33]. If the vestiges of these factories are segregated symmetrically to daughter cells like UBF, attachments – and so gene activity – will be inherited by those daughters.

What role might the intermediate-filament-like nucleoskeleton[34,35] play in chromosome structure? This skeleton probably depolymerizes during mitosis to allow chromosome segregation, and so in one sense it cannot be a key structural component. But it could play an essential role in integrating nuclear space, in the same way that cytoplasmic intermediate filaments integrate cytoplasmic space[36]. It would repolymerize between factories and the lamina as cells entered G_1, providing structural – and so functional – contiguity between factories and a solid phase for transcript movement.

If loops do not change their contour length during mitosis, each loop must condense on itself. It is easy to imagine how inactivating a polymerase positioned at the attachment point might collapse a loop. The template must rotate as it passes through a fixed polymerization site[10,15], so that inactivating the polymerase will inevitably alter the rate of supercoiling, and this could collapse the loop. Another mechanism is based on the properties of 'tensegrity' structures popularized by Buckminster Fuller[37]. Consider a loop of rigid beads strung along an elastic string (e.g. nucleosomes on a loop). Pulling on, and so tensioning, the string – as a polymerase might do during interphase – extends the loop away from the base. Relaxing the tension – like inactivating the polymerase during mitosis – allows the beads to be compacted easily. Then loop compaction in mitosis is an inevitable consequence of inactivating the polymerase and relieving loop tension. This effect may underlie the compaction induced by inhibitors of transcription in lampbrush loops and Balbiani rings[38,39].

Probabilities, domino theory and differentiation

Consider how an inactive gene in the β-globin locus might be

activated. Initially, in the middle of a group of heterochromatic loops, it is remote from factory-bound polymerases. Promoters in peripheral loops have the highest probability of attaching to a factory and might do so when the concentration of some key activator or repressor changes early during haemopoiesis. Transcription then pulls a loop past a fixed polymerase; the resulting movement and/or tensioning opens it up. This opening in turn increases the probability of attachment of a promoter in the next loop in the group. This promoter might be in the locus-controlling region[40], so now adjacent promoters in the β-globin locus become more closely tethered to a factory and so can compete more effectively for polymerases. Which one does so the most successfully will again depend upon the concentration of bound factors and the length of the tether. According to this view, the transcriptional opening of one heterochromatic loop opens another and then proximity and affinity for the factory determine which gene in a locus is transcribed. During this process, LCRs (or enhancers) and the promoters that they control – which may be separated by thousands of base-pairs on a chromosome – are inevitably brought into close proximity by attachment to the same factory.

Transcription and replication

Transcription factories also directly organize sites of replication. In mammalian cells, replication initiates in ~150 foci; as cells progress through S phase they concentrate around nucleoli and the nuclear periphery before dense heterochromatin is replicated in a few large foci at the end of S phase[41]. These foci appear in the electron microscope as dense bodies strung along a nucleoskeleton[42]. Pulse-labelling shows that nascent DNA is extruded from these dense bodies, implying that DNA synthesis occurs as the template slides through DNA polymerases fixed in the body. A single replication fork could not incorporate sufficient label to be detected by either light or electron microscopy, and ~40 forks must be active per focus. Therefore these replication 'factories' also contain many active polymerases.

As transcriptionally active genes are generally replicated before inactive genes, we might expect that some sites of transcription would overlap sites of replication at the beginning of S phase. However, the overlap is better than expected; all replication sites transcribe and all transcription sites replicate[43]. Even later during S phase when heterochromatin – which is widely assumed to be transcriptionally inert – is duplicated, replication sites are still sites of transcription. These results point to a functional relationship between replication and transcription, with the transcription factories seeding assembly of replication factories.

Conclusions

That RNA polymerase can polymerize up to 50 nucleotides per second in an order exactly defined by the template is amazing. I have argued that it (plus associated transcription factors) also manages the structure of the chromosome loop and that groups of polymerases in factories organize clusters of loops. During interphase, the factories are strung along the skeleton and seed assembly of replication factories; during mitosis, the skeleton disappears and transcription factories compact to give the chromomeres of the chromosomal axis. Note that many of these predictions are directly testable: for example, it should be possible (1) to identify discrete sites of Br-UTP incorporation by immunoelectron microscopy and (2) to purify transcription factories containing many polymerases. The RNA polymerizing complex is then both a player and a manager of the chromosome loop. Loop management can take one of two forms, either through alterations in supercoiling and compaction without change in contour length, or through changes in contour length by altering the attachment point. Chromosomes in different tissues in the body would have very different constellations of attachments, and even attachments within a single tissue type would change from moment to moment. RNA polymerase is then a truly remarkable enzyme that doubles as a structural protein; as it transcribes the template, it inevitably changes loop structure. Structure and function are truly intermixed.

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Peter R. Cook is at the CRC Nuclear Structure and Function Research Group, Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK.