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Transcription by an immobilized RNA polymerase from bacteriophage T7 and the topology of transcription

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ABSTRACT

It is often assumed that a polymerase moves along the template as it synthesizes RNA. However, a polymerase that tracks along a helical strand will generate a transcript that is entwined about the template. No such interlocking results if the polymerase is immobile and the template moves past it. Therefore we investigated whether immobilization inhibits the RNA polymerase of T7 bacteriophage using a hybrid protein, in which the polymerase is connected through a peptide linker to an immobilizing domain, which in turn was attached through an antibody to protein A 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 about fivefold. However, when re-initiation was eliminated by removing excess template, immobilization was found to have little effect on the rate of elongation. Perhaps the untwining problem is sidestepped in vivo by immobilizing the polymerase.

INTRODUCTION

Transcription of a double-helix poses various topological problems. One concerns circular or looped templates (1); another is the interlocking of template and transcript that results if the polymerase tracks along a helical strand, as in current models (eg 2). 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).

Model 1 in Fig 1 involves a mobile polymerase both rotating about and translocating along a static template. Then the polymerase, plus nascent transcript and any attached ribosomes (in bacteria) or spliceosomes (in eukaryotes) must all thread through the centre of a circular or looped template, once for every ten base-pairs transcribed. As the diameters of ribosomes and spliceosomes roughly equal those of small plasmid circles condensed by supercoiling, this seems improbable. Moreover, the resulting transcript and template are intertwined, but we have no mechanism for untwining them. These 'threading' and 'untwining' problems seem insuperable, making model 1 unlikely. Model 3 faces the same intractable problems.

These problems are sidestepped if DNA rotates instead of the polymerase. In model 2—the 'twin-supercoiled-domain' model (1)—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 (eg 3–6), this model faces the problem of preventing the polymerase from rotating whilst allowing it to translocate. Even one accidental rotation—which is likely when the transcript is short and frictional drag limited—would yield an entwined transcript. Rotation might be restricted if the polymerase deformed the template into a large apical loop (7), but it again seems unlikely that this could eliminate any rotation throughout a long transcription unit. 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 (8,9). 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' (10) or in the special case of a modified phage enzyme provided with an artificial anchorage point (11).

We have now investigated whether immobilization inhibits the RNA polymerase of bacteriophage T7, chosen because its soluble form is one of the most active known (12). We compare the activity of the enzyme free in solution and attached to plastic beads. Although the free enzyme initiates more efficiently, the two forms elongate at similar rates.

MATERIALS AND METHODS

The hybrid gene

The gene for a tripartite protein, the maltose-binding protein of E. coli connected through a protein linker of 13 amino acids (the last 4 containing a cleavage site for the protease, factor Xa) to the T7 RNA polymerase, was prepared (13). The vector pMAL-c (supplied with the protein fusion and purification kit from BioLabs) contains a modified *malE* gene, which encodes the maltose-binding protein lacking a signal sequence so that on

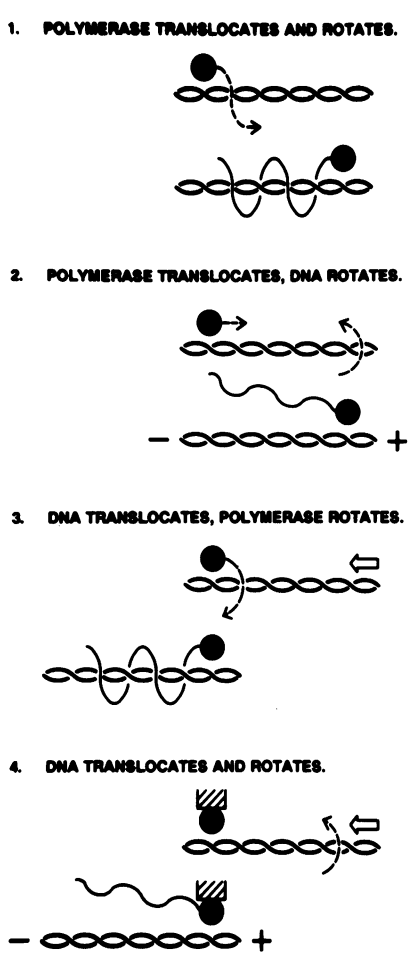

Fig 1. Models for transcriptional elongation involving mobile or static polymerases (black circles) and double-helical templates. Upper panels in each model indicate initial relative positions; arrows show subsequent movements. Lower panels illustrate final positions after transcription. + and − indicate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

expression the protein is not exported to the periplasm (14). The *malE* gene is transcribed from the IPTG-inducible P_{tac} promoter, so allowing inducible over-expression of a toxic protein like RNA polymerase. pMAL-c DNA was cut with *Bam*HI and *Hind*III, treated with alkaline phosphatase, and ligated with an insert coding for the Xa cleavage site and the T7 polymerase. The insert was obtained by performing a polymerase chain reaction (25 cycles; 60°C for 2sec, 72°C for 90sec and 94°C for 30sec; 1ng template) using two primers complementary to the ends of the T7 RNA polymerase gene contained in plasmid pAR 1219 (15). One primer (5'CCCCGGATCCATCGAAGGTCGT*ATG*AACACGATTAACATCGC)—which contained the Xa site—encoded a 5' C-rich clamp, a *Bam*HI site (underlined), the recognition sequence for factor Xa (shown **bold**; the corresponding amino acid sequence is IEGR), followed by the first 20 nucleotides at the start of the T7 polymerase gene (ie positions 3171−3190 in the DNA sequence; 16). The ATG coding for the initiating methionine is shown in *italics*. The second primer (5'CCCCAAGCTTCCGAGTCGTATTGATTTGGC) encoded a 5' C-rich clamp, a *Hind*III site (underlined), the dinucleotide CC (incorporated into pAR 1219 during cloning), followed by 18 nucleotides identical to the non-coding strand at the 3'end of the polymerase gene (ie positions 5841−5824). The product of the chain reaction was cut with *Bam*HI and *Hind*III, ligated with vector DNA, and the resulting recombinant used to transform a protease-deficient *E. coli*, strain UT5600, used because the polymerase is sensitive to proteolysis (17). DNA of the resulting plasmid, pFG4, had the expected restriction map.

pFG4 should contain a recombinant gene with the strong P_{tac} promoter, *malE* translation initiation signals, codons 1 and 27−392 of *malE* (mature *malE* product is encoded by codons 27−396; 18), 13 codons in the linker (the last four encoding the Xa-recognition sequence), the complete coding sequence for T7 RNA polymerase (positions 3171−5819), a truncated *lacZα* gene and strong *rrnB* transcriptional terminators (Fig 2A). pFG4 also encodes the *lacI* gene, so the P_{tac} promoter is usually inactive but, on induction with IPTG, a tripartite hybrid protein (hp4) is expressed.

The hybrid protein and maltose-binding protein

Procedures for growing bacteria containing pFG4 or pMAL-c, inducing expression, purification on amylose columns and analysis by SDS-PAGE were as described in the protein fusion and purification kit (BioLabs; 19). Protease inhibitors (PMSF, leupeptin, TPCK, pepstatin) were added to buffers used for lysing bacteria and columns; PMSF was added to dialysis buffers.

RNA polymerases

Three different preparations containing T7 RNA polymerase activity were used: (1) A pure commercially-supplied preparation (Boehringer; ~250ng/μl; 20 units/μl). (2) A crude extract obtained by sonicating bacteria over-expressing hp4, adding NaCl to 0.5M and spinning to remove debris at 9000g for 30min. This extract was flash frozen and stored at −70°C; immediately prior to use it was spun (microcentrifuge; 4°C; 10min) to remove aggregates. (3) Purified hp4 (~300ng/μl) was obtained by passing crude extract over an amylose column, eluting with maltose, dialysing and concentrating by filtration. Controls showed that pure hp4 and the polymerase from Boehringer had roughly equivalent specific activities; Table I gives relative activities of different polymerases.

Sera

Rabbit sera were obtained from pre-immune animals and those immunized with 4 successive injections (10-day intervals) of purified maltose-binding protein (20).

Binding of hybrid protein to protein A beads

Acrylic beads to which protein A was covalently attached (150μm; Sigma) were suspended in an equal vol of phosphate-buffered saline. 1 vol crude extract containing hp4 was incubated (ice; 1h) with 2 vol immune rabbit serum. Then 1.3 vol beads were added and tumbled (4°C; 1h) before unbound material was removed by washing 3× with >10 vol saline then 2× with >10 vol KGB buffer (21; 100mM potassium glutamate, 25mM Tris acetate pH 7.6, 10mM magnesium acetate and 0.25mM dithiothreitol). Beads were washed with conservation of initial vol by adding >10 vol ice-cold KGB, mixing, spinning (1 sec; microcentrifuge) and removing the same vol as was added.

Transcription reactions

Transcription reactions (450μl) generally contained ATP, CTP and GTP at 500μM in KGB, plus 5−50nM [α^{32}P]-UTP (~3000Ci/mmol; Amersham). The template containing the T7

Table I. Relative initial rates of transcription measured in the absence of heparin.

	3 min			30 min		
	−Xa	−Xa	+Xa	***Ratio (+Xa/−Xa)***	−Xa washed	+Xa washed
	a	b	c	***d***	e	f
1. Pure T7 pol	1.0	0.26	0.10	***0.4***	–	–
2. Pure hp	1.0	0.77	0.19	***0.3***	–	–
3. Beads/pre-imm + pure T7 pol	1.0	0.71	0.21	***0.3***	0.01	0.01
4. Beads/pre-imm + pure hp	1.0	0.85	0.18	***0.2***	0.05	0.05
5. Beads/imm/hp	1.0	1.13	1.47	***1.3***	0.32	0.11

Various forms of polymerases were incubated for 3 or 30min at 20°C (−/+ Xa), initial rates of transcription measured (as in Fig 6A) and expressed relative to the rate in column a. Values are averages of at least 2 experiments.
Lines 1 and 2: no beads added.
Lines 3 and 4: beads treated with pre-immune serum, washed and then either pure T7 polymerase or pure hybrid protein added.
Line 5: crude extract incubated with immune serum, immune complexes bound to beads and beads washed.
In columns e and f, beads were washed immediately prior to transcription. Column d gives ratio of column b:c.

Table II. Relative rates of elongation measured after suppressing re-initiation with heparin.

	3 min				30 min		
	−hep −Xa	+hep −Xa	+hep −Xa	+hep +Xa	***Ratio (+Xa/−Xa)***	+hep −Xa washed	+hep +Xa washed
	a	b	c	d	***e***	f	g
1. Beads/pre-imm + T7 pol	1.0	0.47	0.20	0.25	***1.3***	-	-
2. Beads/pre-imm + hp	1.0	0.52	0.16	0.25	***1.6***	-	-
3. Beads/imm/hp	1.0	0.40	0.12	0.62	***5.2***	0.06	0.03
4. Beads/imm/hp, washed	1.0	0.85	0.62	0.80	***1.3***	0.53	0.23

Various polymerases were incubated for 3min to form initiated complexes and then for 3 or 30min at 20°C (−/+ heparin, −/+ Xa); then initial rates of elongation were measured and expressed relative to the rate in column a. Values are averages of at least 2 experiments.
Lines 1 and 2: beads treated with pre-immune serum, washed and then either pure T7 polymerase or pure hybrid protein added.
Line 3: immune serum and crude extract were mixed, first together and then with beads and beads washed. Averages of 7 experiments.
Line 4: as line 3, except that washed beads were incubated for 3min to form initiated complexes, re-washed and then incubated for 3 or 30min at 20°C (−/+ heparin, −/+ Xa), as in Fig 6B. Averages of 3 experiments.
Columns f,g: beads washed at the end of 30min incubation (as in Fig 6B, curves 5,6) immediately prior to elongation in [α^{32}P]-UTP. Column e gives the ratio of column c:d.

promoter—bluescript plasmid, pBSks+ (Stratagene; >75% supercoiled)—was present at ~25ng/μl during reactions or during synthesis of initiated complexes containing 7 nucleotide-long transcripts. Heparin (Sigma grade II) was added during pre-incubations to 20μg/ml and factor Xa (BioLabs or Danex Biotek) to 100μg/ml. Care was taken to resuspend beads uniformly just before pipetting.

A typical experiment (eg Table I, line 5) involved incubating beads with attached hybrid protein (150μl; 3 or 30min; 20°C) prior to equilibration (3min; 37°C) before initiating transcription by addition of 300μl triphosphates (including [α^{32}P]-UTP); sometimes Xa was added at the beginning of the 30min. Beads with attached hybrid protein were washed thoroughly and resuspended in ice-cold KGB to give a 7% suspension. 500μl aliquots were dispensed immediately, beads pelleted and 380μl supernatant removed. 15μl DNA (250ng/μl) was added, followed by 15μl KGB −/+ Xa (1mg/ml) and incubated (3 or 30min; 20°C). Some samples were washed (2×; 10vol ice-cold KGB) during the last 5min of the 30min incubation. Next, samples were equilibrated (37°C; 3min) before 300μl ATP, CTP, GTP plus 2−20μCi [α^{32}P]-UTP in KGB were added. 80μl samples were withdrawn after 0.25, 5, 10 and 15min, and mixed with 150μl 1%SDS to stop the reaction. Duplicate 80μl samples of this stopped mixture were spotted on to 25mm GF/C circles (Whatman), the circles extracted successively with 5% trichloroacetic acid, ethanol and ether before drying and counting in a scintillation counter. Initial rates were estimated between 15sec and 5min. Label concentration was adjusted to give >1000cpm (usually ~10,000cpm) above background per sample after 5min, equivalent to >2fmole UMP incorporated. Where samples were washed after 25min incubation, heparin was added to the same final concentration (ie 6.7μg/ml) during transcription as unwashed samples.

Experiments (eg Table II, line 3) involving initiated complexes (formed with ATP, CTP and GTP, but not UTP) were as follows. Beads were pelleted, supernatant removed, DNA added as above and the mixture equilibrated (37°C; 3min) before 15μl containing 5mM ATP, CTP and GTP in KGB added. After initiation (3min;

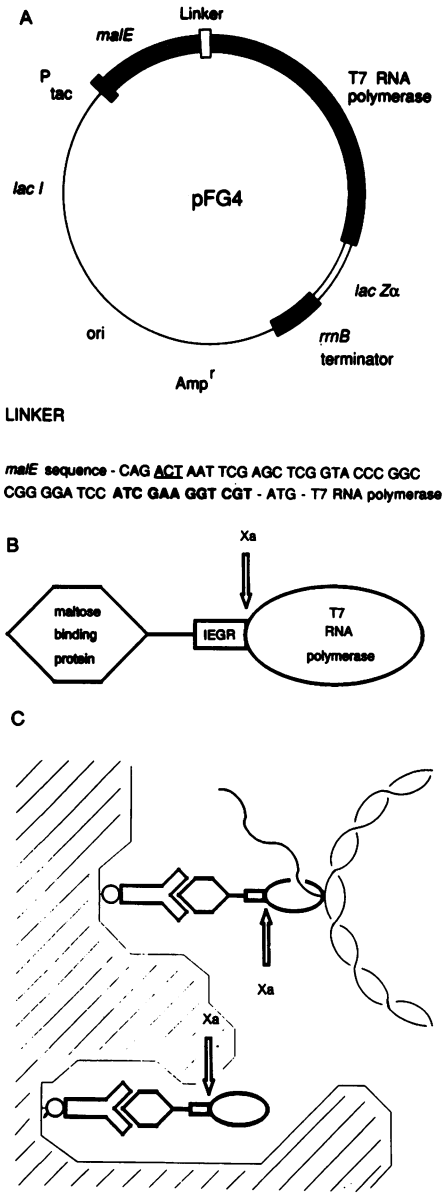

Fig 2. Immobilizing T7 RNA polymerase. A. The structure of the plasmid encoding the hybrid polymerase and the linker sequence. The underlined ACT is the 392nd codon of *malE*, the region in bold encodes the Xa recognition sequence (IEGR) followed by the ATG coding for the first amino acid of the polymerase. B. Inducible transcription from P_{tac} and subsequent translation forms a hybrid protein, with maltose-binding protein and polymerase domains, connected through a peptide linker containing the Xa-cleavage site, IEGR. C. Two hybrid proteins are attached via antibodies (Y-shaped structures) to protein A (circles) covalently attached to plastic (hatched area). The upper hybrid protein has bound template and transcribed it; the lower one is almost inaccessible to template. Xa-treatment releases both polymerizing moieties.

37°C), heparin and Xa were added if needed, the volume made up to 150μl and samples incubated (3 or 30min; 20°C). After equilibration (3min; 37°C), triphosphates plus label were added as above. Washed initiated complexes (eg Table II, line 4 and Fig 6B) were prepared as above by using larger volumes and washing (2×; 10vols ice-cold KGB) after incubation (3min; 37°C) with the three unlabelled triphosphates. Then beads were immediately resuspended in KGB to give a 7% solution, 500μl aliquots dispensed, beads pelleted and most supernatant removed.

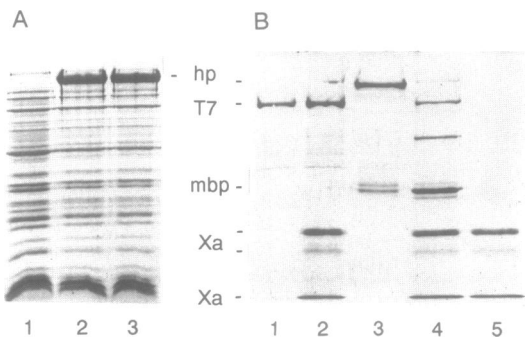

Fig 3. Crude (A) and purified (B) hybrid protein analyzed on acrylamide gels. A. Extracts from uninduced (lane 1), induced (lane 2) and induced and sonicated bacteria (lane 3) were run on a 12% gel, stained with Coomassie blue and photographed. hp: hybrid protein. B. Authentic T7 polymerase and pure hybrid protein were treated with Xa (100μg/ml), run on a 10% gel and photographed. Lane 1: T7 RNA polymerase. Lane 2: T7 polymerase treated with Xa. Lane 3: hybrid protein. Lane 4: hybrid protein treated with Xa. Lane 5: Xa. Hybrid protein (hp), contaminated by maltose-binding protein (mbp), is cut by Xa into authentic T7 polymerase (T7), maltose-binding protein and a band of intermediate size.

KGB −/+ heparin and −/+ Xa was added to give a final volume of 150μl. Some samples were incubated (30min; 20°C) before equilibration and addition of triphosphates plus label, as above.

Transcripts were sized on denaturing 6% polyacrylamide gels and autoradiographs prepared (13).

Dissociation of hybrid protein from beads

Dissociation rates were measured under conditions used for transcription. Hybrid protein was bound to beads as above and after addition of template and four unlabelled triphosphates to 500μM, samples were incubated for different periods, pelleted, washed (2×; >10vol ice-cold KGB) and electrophoresis sample buffer added. After electrophoresis on 10% polyacrylamide gels and staining with Coomassie blue, the amount of hybrid protein remaining was determined by quantitative densitometry. The dissociation rate was unaffected by heparin.

RESULTS

Strategy

Treatments used to attach a pure polymerase to a surface (eg adsorption or chemical cross-linking) might alter its specific activity, making investigation of any additional effects due to immobilization difficult, if not impossible. And as we do not yet know its three-dimensional structure, we might inadvertently attach it through its active site. Therefore, we connected an immobilizing domain—a maltose-binding protein—to the polymerase (Fig 2A,B). Such a hybrid protein can be attached to amylose beads, allowing both purification and immobilization. The attached protein can be released from amylose by addition of maltose, so that bound and free forms can be compared. As the two domains of the hybrid protein are connected by a linker that can be cut by the specific protease, factor Xa, authentic T7 polymerase can be released. Unfortunately, the hybrid protein dissociated from amylose baked on the bottom of a microtitre well with a half-life of only 5min, making kinetic analysis difficult. [Nevertheless, elongation rates of the free and bound forms were similar (not shown).] Therefore we immobilized the hybrid protein indirectly through a protein A-antibody complex

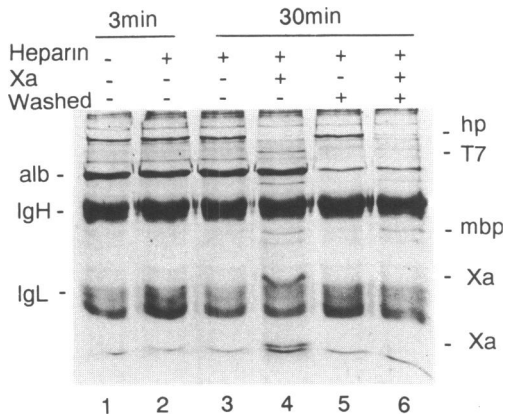

Fig 4. Detachment by Xa of the polymerase moiety from beads during a typical transcription reaction. Hybrid protein was bound to beads and template plus triphosphates lacking UTP added to generate initiated complexes containing 7-nucleotide-long transcripts. After adding heparin to most samples to prevent re-initiation, some reactions were stopped after 3min. The rest were incubated for 30mins (−/+ Xa; 100μg/ml) before some beads were washed and reactions stopped. The polypeptides present were run on a gel and the stained gel photographed. A complex set of polypeptides are initially bound to beads (lane 1), including the hybrid protein (hp), plus heavy and light immunoglobulin chains (IgH and IgL) and albumin (alb). Treatment with Xa for 30min (lane 4) cuts the hybrid protein into authentic T7 polymerase (T7), maltose-binding protein (mbp) and a band of intermediate size. Washing beads removes the T7 moiety and Xa but not the maltose-binding moiety (lane 6).

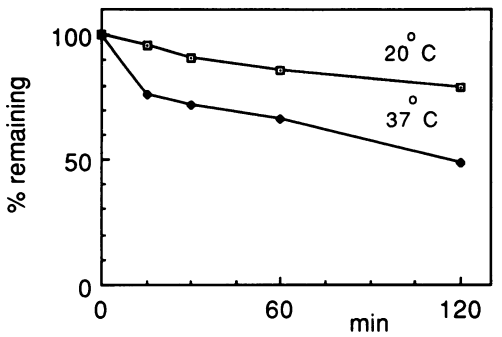

Fig 5. Dissociation of bound hybrid protein from beads. Sonicated extracts of bacteria containing hybrid protein were incubated with serum from a rabbit immunized with maltose-binding protein and the resulting immune complexes bound to protein A beads and washed thoroughly. After incubation in complete transcription mixture for different times at 20 or 37°C, beads were washed and any polypeptides remaining attached analyzed on gels, as in Fig 4. The percentage of hybrid protein remaining bound relative to controls at zero time was estimated by densitometry (means of at least 3 experiments).

to 150μm acrylic beads, using antibodies directed against the maltose-binding protein (Fig 2C). Transcription of a superhelical template by this bound polymerase and the authentic enzyme released from beads after cleavage with factor Xa were then compared. This antibody-based strategy has the advantage that in future transcription factors can be immobilized with polymerases using appropriate antibodies.

The hybrid polymerase

A synthetic gene coding for the hybrid protein was made, inserted into a plasmid (Fig 2A) and, after expression in bacteria, a polypeptide with the expected size was obtained (Fig 3A, lane 2). Sonication and pelleting to remove debris then led to a 'crude extract' (Fig 3A lane 3) from which relatively pure hybrid protein was prepared on an amylose column (Fig 3B lane 3). Factor Xa cut this into two polypeptides with the size of authentic T7 polymerase and the maltose-binding protein, plus a third of intermediate size (Fig 3B lane 4; we do not know the reason for this novel cleavage). Commercially-supplied T7 RNA polymerase was not cleaved by Xa (Fig 3B lanes 1,2). There was no cleavage into a form with an Mr of ~75,000, known to have altered activity (22).

Immobilization

An immune complex was formed by mixing the 'crude extract' with a polyclonal serum directed against pure maltose-binding protein and mixed in turn with beads to which protein A was covalently bound. After washing thoroughly, hybrid protein attached to beads through the antibody-protein A bridge was recovered. No hybrid protein bound using pre-immune serum (not shown).

Fig 4, lane 1 illustrates the polypeptides associated with beads after such an immunopurification. The hybrid protein (hp) was a minor component; major components are the serum-derived heavy and light immunoglobulin chains (IgH, IgL), plus albumin (alb). Xa-treatment cut the hybrid protein into the authentic T7 polymerase and maltose-binding moieties, plus the band of intermediate size (Fig 4 lane 4). Quantitative densitometry showed that routinely >90% of bound hybrid protein was cut. Unlike unspecifically-bound albumin, little hybrid protein was washed away from beads (Fig 4 lane 5); as expected, washing Xa-treated beads removed Xa plus the free polymerase, some of the band of intermediate size, but none of the maltose-binding moiety (Fig 4 lane 6).

Unfortunately, the hybrid protein slowly eluted from beads (Fig 5), so initial rates of transcription were routinely measured 3 and 30min after washing to monitor the effects of dissociation. We also incubated beads for 6min at 37°C followed by 25min at 20°C to allow low-affinity antibodies to dissociate, prior to rewashing and immediate assay (eg Fig 6B).

These experiments demonstrate that the hybrid protein is relatively stably bound and that treatment with factor Xa frees the authentic polymerase (Figs 4,5).

Initial rates of transcription in the absence of heparin

Initial rates of transcription from the T7 promoter carried on a ~3kbp supercoiled template are measured by incorporation of [α^{32}P]-UTP into acid-insoluble material. To conserve label, no unlabelled UTP was added so its concentration is sub-optimal and synthesis inefficient. Each step during transcription (eg initiation, elongation and termination) is complicated and still incompletely understood (eg 12,23). However, theory suggests that immobilization would particularly inhibit initiation: polymerase-promoter interactions become essentially two dimensional on a surface, rather than three-dimensional in solution. Direct effects of Xa-cleavage must also be distinguished from indirect effects of adding a carrier protein; Xa both stimulates or inhibits commercially-supplied T7 RNA polymerase, depending on the conditions (see below). [Note that comparative experiments showed that pure hybrid protein and the commercially-supplied enzyme had roughly equivalent specific activities.]

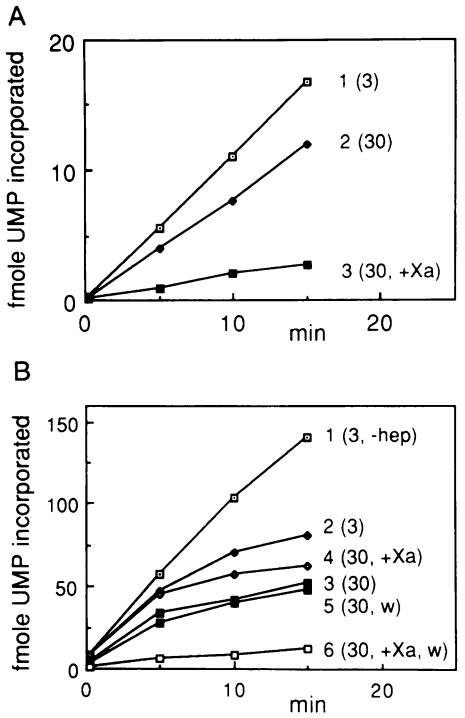

Fig 6. Transcription by hybrid protein, (A) free and in the absence of heparin and (B) bound in the presence of heparin. A. Pure hybrid protein was added to template, pre-incubated for 3 or 30min −/+ Xa, triphosphates added and the rate of incorporation of [α^{32}P]-UTP into RNA determined. Curve 1: 3min pre-incubation −Xa. Curve 2: 30min pre-incubation −Xa. Curve 3: 30min pre-incubation +Xa. B. Hybrid protein bound to beads was incubated with template and three triphosphates to form initiated complexes. Beads were then washed to remove unbound template, incubated for 3 (curves 1 and 2) or 30min (curves 3−6) −/+heparin (hep) or Xa, and the rate of incorporation determined. Some beads were washed (w) immediately prior to incorporation. Curve 1: 3min, −hep, −Xa. Curve 2: 3min, +hep, −Xa. Curve 3: 30min, +hep, −Xa. Curve 4: 30min, +hep, +Xa. Curve 5: as curve 3, but washed. Curve 6: as curve 4, but washed.

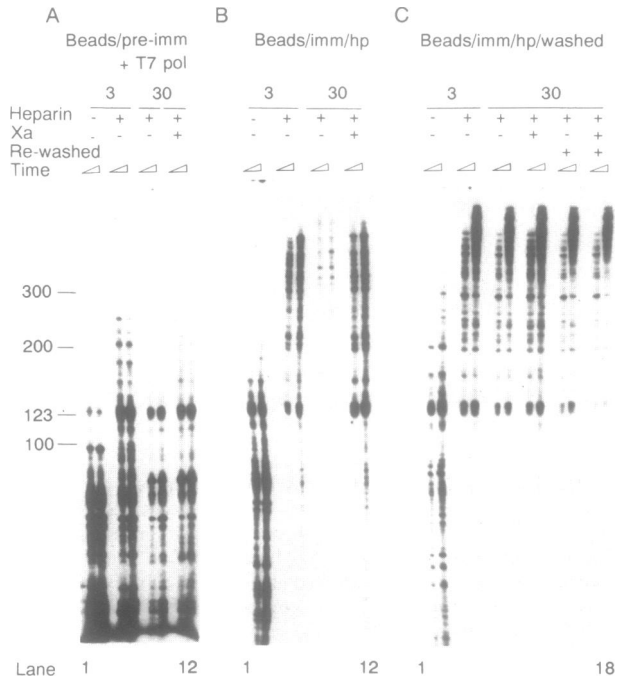

Fig 7. The size of transcripts synthesized by different polymerases, after forming initiated complexes. Equal vol reactions containing labelled transcripts were run on denaturing gels and autoradiographs prepared. Samples were withdrawn at 0.25, 5 and 15min (indicated by triangles), giving three tracks per reaction. Nucleotide sizes are indicated on the left. (A) Beads coated with pre-immune antibodies plus added T7 RNA polymerase. Beads were incubated with pre-immune serum, washed, pure T7 polymerase added and initiated complexes formed before incubation for 3 or 30min at 20°C (−/+ heparin, −/+ Xa) and elongation in [α^{32}P]-UTP. (B) Hybrid protein bound to beads through immune antibodies. Crude extract was incubated with immune serum and immune complexes bound to beads. After washing, initiated complexes were formed and then treated as in A. (C) As B, but initiated complexes attached to beads were washed to remove excess template prior to incubation for 3 or 30min.

Fig 6A illustrates a typical reaction. Template and hybrid protein were mixed (without beads) and pre-incubated for 3 at 20°C; transcripts then initiate correctly at the T7 promoter (see below) and label is incorporated linearly (curve 1). Some activity is lost after pre-incubation for 30min (curve 2) and factor Xa enhances this loss (curve 3). Initial rates were measured between 15sec and 5min and normalized relative to the rate in curve 1; Table I, line 2 gives the averages of several experiments. Factor Xa inhibits the authentic polymerase similarly (Table I, line 1; column d gives the rate+Xa/rate−Xa); presumably it withdraws both polymerases from the reaction. Xa also inhibited both enzymes in the presence of beads treated with pre-immune serum (lines 3,4; column d). Washing prior to transcription removed both these free activities (lines 3,4; columns e,f). In contrast, incubating bound protein for 30min increased activity (line 5, cf columns a,b) and Xa-treatment stimulated this further (line 5, column d). After 30min without Xa, washing prior to assay removed some—but not all—activity (line 5, cf columns b,e). Washing Xa-treated beads removed nearly all activity, presumably because it had been detached (line 5, cf columns c,f).

These results can be explained in several ways. Bound protein might be completely inactive, with any incorporation resulting from dissociated enzyme. Then, extending the dissociation time to 30min should increase the initial rate (as it does) and Xa-treatment should dramatically stimulate it further (as it does only 1.3×). Alternatively, bound and free forms might elongate equally, with the free form initiating better. This is to be expected: template might have only limited access to bound polymerase (Fig 2C, upper complex), with some being inaccessibly buried in crevices in the porous beads (Fig 2C, lower complex); cleavage would release both types into the supernatant where most template is to be found. Put in another way, cleavage both increases the total number of potentially-active polymerases and template availability. Then results in Table I, line 5 are explained as follows. After 30min, more template diffuses into crevices, giving the higher rate (columns a,b). Xa-treatment releases polymerase, dramatically increasing initiation which, though dampened by the inhibitory effect of adding protein (lines 1−4, column d), nevertheless leads to a net stimulation (line 5, column d). Therefore we measured elongation rates under conditions where initiation was suppressed.

Elongation rates in the presence of heparin

Heparin inhibits polymerases by trapping free enzyme in unproductive complexes; initiated complexes are relatively immune to inhibition (22). Therefore hybrid protein was bound and transcription initiated by adding ATP, CTP and GTP, but not UTP. Initiated complexes with 7 nucleotide-long transcripts

are formed on our template, as the first U is incorporated at position eight. Heparin is now added to trap un-initiated polymerase, the complexes incubated with and without Xa and labelled UTP added.

Heparin has similar effects on free T7 polymerase and hybrid protein (assayed in the presence of beads coated with pre-immune antibodies); the rate is roughly halved as re-initiation is inhibited (Table II, lines 1,2; columns a,b). After 30min, further activity is lost (lines 1,2; column c). Heparin similarly affects bound enzyme (line 3, columns b,c). Now Xa-treatment slightly stimulates both free T7 polymerase and free hybrid protein (lines 1,2; column e); presumably initiated complexes are more resistant to disruption by added protein than free enzyme. Xa-treatment stimulates the bound enzyme even more (ie 5.2×; line 3, column e).

This fivefold stimulation is consistent with free polymerase elongating at five times the rate of bound enzyme. If so, transcripts generated after Xa-treatment should be five times longer, given that heparin should prevent re-initiation. However, they are not (see below). Therefore re-initiation was not **completely** inhibited, probably because the protein-rich beads bind heparin and reduce its effective concentration. In other words, Xa-treatment releases so much enzyme able to initiate more efficiently that even a small fraction escaping inactivation by heparin causes a net stimulation, obscuring any effects on elongation.

Fortunately, re-initiation can be eliminated completely by washing beads to remove all unbound template prior to assay, so allowing a decisive test of the effects of immobilization on elongation. This also selects for tightly-bound hybrid protein; any bound by low-affinity antibodies will dissociate during initiation (6min at 37°C) and be washed away.

Initiated complexes bound to beads were prepared as before, washed free of unbound template and transcription started by adding labelled UTP (Fig 6B curve 1). Heparin now only slightly reduced the initial rate, as all template is in initiated complexes (Fig 6B curve 2). [Nevertheless, heparin withdraws some enzyme from initiated complexes as the rate decreases progressively over 15min.] 30min incubation reduced the rate further (Fig 6B curve 3) and Xa stimulated it only slightly (Fig 6B curve 4). This stimulation was usually 1.3× (Table II, line 4, column e), exactly that found with free T7 polymerase (line 1, column e). Therefore, when re-initiation is eliminated by removing all unbound template, there is only a 1.3× difference in elongation rate of bound and free forms. This difference is entirely attributable to the effects of adding carrier protein.

Most activity found after 30min without Xa remains associated with beads, as washing removes little of it (Fig 6B, cf curves 3,5; Table II, line 4, cf columns c,f). In contrast, Xa-treatment detaches activity so it can be washed away (cf curves 4,6; Table II, line 4, columns d,g). This provides decisive evidence that activity seen in the absence of Xa results from bound enzyme.

Transcript sizes

At the low UTP concentration used, transcription is inefficient and transcripts stall or terminate prematurely wherever UTP is required. Many do so 123 nucleotides downstream from the promoter, where four consecutive uridines are incorporated. Most transcripts synthesized by initiated complexes of pure T7 polymerase (in the presence of beads coated with pre-immune antibodies) are <123 nucleotides long (Fig 7A lanes 1−3). Heparin stimulates the formation of longer transcripts and depresses the formation of short ones by inhibiting re-initiation (lanes 4−6); Xa-treatment has little effect (lanes 7−12). Although there are differences in detail, all forms of unbound enzyme give essentially the same patterns, whether initiated complexes are preformed or not: these include pure T7 polymerase in the absence of beads and pure hybrid protein −/+ beads coated with pre-immune antibodies (not shown).

Bound hybrid protein gives roughly similar patterns (Fig 7B). In the absence of heparin, initiated complexes again synthesize short transcripts of <123 nucleotides (lanes 1−3) and heparin stimulates the formation of longer ones (lanes 4−6). 30min incubation reduces the total amount of synthesis without much effect on the pattern (lanes 7−9) and Xa-treatment stimulates incorporation fivefold (Table II, line 3, column e) **without** the synthesis of fivefold longer transcripts expected if free enzyme elongated more efficiently and heparin completely inhibited re-initiation: rather, they are, on average, slightly smaller (lanes 10−12). Therefore the fivefold stimulation must result from increased initiation by released polymerase on extra templates in the supernatant. This Xa-induced re-initiation is reflected by the increased proportion of 123-nucleotide-long transcripts (cf lanes 8,9 and 11,12).

That transcripts generated by free and bound polymerases are equally long is confirmed using bound initiated complexes, washed free of excess template (Fig 7C). In heparin, the patterns are similar, whether polymerase is treated with Xa or not. This confirms that elongation is unaffected by immobilization.

DISCUSSION

Our main aim was to determine whether immobilizing an RNA polymerase inactivated it. A hybrid protein, part maltose-binding protein and part T7 RNA polymerase, was attached through an antibody bridge to protein A covalently attached to plastic beads. Then the activities of bound and free forms were compared by releasing authentic T7 RNA polymerase using factor Xa. Our results are simply interpreted if immobilization dramatically reduces initiation without much effect on elongation.

Direct effects of immobilization were disentangled from any indirect effects by comparing the activities of free polymerases (ie commercially-supplied T7 RNA polymerase or pure hybrid protein) with those of the bound hybrid protein. When re-initiation was allowed, Xa-treatment inhibited free polymerases, but stimulated the bound enzyme (Table I, column d). But when re-initiation was completely prevented, only slight differences attributable to the effects of carrier protein were detected (Table II, column e). We might expect initiation to be particularly inhibited by immobilization because polymerase-promoter interactions, normally three-dimensional in solution, are restricted to the two dimensions of a surface.

The conclusion that elongation is unaffected by immobilization depends on the assumption that most activity seen is indeed due to bound enzyme and not to a fraction that has eluted from beads (Fig 5). Experiments described in Fig 6B and Table II, line 4 make it unlikely that only a dissociated fraction is active, with attached protein being completely inactive. Here initiated complexes were formed at 37°C and then washed to remove both excess template that might be used for re-initiation and any protein bound by low-affinity antibodies. If inactive bound complexes then dissociated to give the activity seen, re-washing should have removed them, but it did not. In contrast, Xa-treatment generated activity that could be washed away. Furthermore, the length of

the transcripts generated by the bound and free forms was similar (Fig 7). These results are simply explained if bound and free forms elongate equally efficiently.

These studies all involved transcription limited by the low UTP concentration. Therefore we repeated experiments described in Table II, lines 3 and 4, using 25μM UTP: Xa now stimulated rates 3.8× and 1.2× respectively. After 5min synthesis, most transcripts were >2000 nucleotides long, with many longer than the circular template (not shown). These results, then, are not confined to low UTP concentrations.

Our results are consistent with those of Schafer et al. (24) who observed (in the light microscope) transcription by single molecules of the bacterial enzyme immobilized on a coverslip; again it elongated as efficiently as free enzyme. They raise the possibility that RNA polymerases are normally immobilized in vivo, thus sidestepping 'threading' and 'untwining' problems (Fig 1). How might they be immobilized? One possibility is that active polymerases have two DNA binding sites (either intrinsically or as dimers), so they can anchor themselves to one piece of DNA whilst transcribing another, as in the bacterial 'nucleoid' (10). [Note that both the T7 and the E. coli enzyme tend to aggregate at physiological salt concentrations (12,25).] Alternatively, polymerases might be immobilized by attachment to a skeleton, as may be the case in eukaryotes (8,9,26). Then, transcription is more like driving a screw (helical DNA) through a fixed nut (the polymerase), whilst a ratchet (a topoisomerase) in the screwdriver releases the torsional strain. This is to be contrasted with the traditional analogy of a locomotive (the polymerase) moving down a track (the template).

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