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Different Populations of DNA Polymerase α in HeLa Cells

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Three different populations of HeLa DNA polymerase α have been distinguished using a novel preparation of chromatin isolated using an isotonic salt concentration, which contains intact DNA. One synthesizes DNA *in vitro* at 85% of the rate *in vivo*, is found only in *S*-phase nuclei tightly associated with the nucleoskeleton and requires unbroken DNA in the form of chromatin as a template: we assume this is the authentic *S*-phase activity. On incubation at 37°C, this activity dissociates from the nucleoskeleton to give a soluble activity that prefers broken templates. This soluble activity is in turn heterogeneous, containing active complexes of about $0{\cdot}75 \times 10^6$ and 3×10^6 M_r. The third activity is also soluble and released by lysing cells at any stage of the cell cycle. It, too, prefers broken templates. The authentic activity is obscured by the soluble ones if broken templates are provided.

1. Introduction

Studies on the enzyme complex involved in replicating nuclear DNA, which contains polymerase α, have been hampered by two major factors. The first is the instability of the enzyme–template complex. As one might expect, antibodies directed against polymerase α bind to nuclei, the presumed site of action of the enzyme (Bensch *et al.*, 1982). However, when cells are fractionated using conventional procedures in which cells are swollen in a hypotonic salt concentration, most of the polymerase activity is found not in the nucleus but in a soluble cytoplasmic fraction (Kornberg, 1974; De Pamphilis & Wassarman, 1980). The second factor concerns the template. Soluble enzymes replicate DNA in chromatin poorly, preferring pure DNA containing many single-strand breaks (i.e. "activated" DNA). Furthermore, it has hitherto proved impossible to isolate chromatin containing unbroken DNA. As a result, a soluble enzyme that replicates an unnatural template is much better understood than the "native" enzyme, which must use an intact chromatin template in the nucleus.

Recently we have described a method for freeing nuclei of most soluble cytoplasmic material using a physiological concentration of salt; the resulting chromatin contains unbroken DNA (Jackson & Cook, 1985). Cells are encapsulated in agarose microbeads to provide them with a protective coat. Enzymes and other small molecules, but not the very much larger chromatin, can diffuse freely through the agarose pores. When the encapsulated cells are lysed in Triton X-100 in an isotonic salt concentration, soluble cytoplasmic material diffuses out of the beads to leave nuclei and a surrounding cytoskeleton. These encapsulated nuclei can be pipetted freely in a variety of conditions without aggregation or breaking their DNA. We have partly characterized a DNA polymerase α activity in such encapsulated nuclei from HeLa cells (Jackson & Cook, accompanying paper). *In vitro* this activity replicates the intact chromatin template at 85% of the rate found *in vivo*. It is found only in *S*-phase nuclei and, as we shall show, replicates added activated templates poorly, if at all. It probably elongates molecules already initiated *in vivo*. As it is associated with the nucleoskeleton (Jackson & Cook, 1986) we call it polymerase α^{nsk}†. In addition, we describe two other activities that prefer broken templates. One, α^{det}, is probably formed when α^{nsk} detaches from the nucleoskeleton on incubation at 37°C and so requires a new template and primer for activity. The other is also a soluble activity, α^{sol}, and is released on lysing encapsulated cells whatever their stage in the cell cycle. We presume that *in vivo* α^{sol} forms a pool of inactive enzyme and when activated it becomes stably associated with the nucleoskeleton (i.e. it becomes α^{nsk}).

† Abbreviations used: α^{nsk}, polymerase α that remains associated with the nucleoskeleton and hence the beads; α^{det}, a soluble polymerase α activity, probably derived from α^{nsk} by detachment from the nucleoskeleton, that appears on incubation at 37°C; α^{sol}, a soluble polymerase α activity, released from cells during lysis; kb. 10^3 base-pairs; BSA, bovine serum albumin.

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2. Materials and Methods

(a) *General*

All general methods are described in the accompanying paper (Jackson & Cook, 1986*b*) and only special variations are given here.

(b) *Cells*

HeLa cells were used throughout and generally labelled with [^{3}H]thymidine (18 to 24 h, 0·05 μCi/ml, 60 Ci/mmol). In some cases cells were labelled with [^{35}S]methionine (10 μCi/ml, 500 mCi/mmol) for 18 h at 37°C in methionine-free minimal essential medium for suspension cultures (Gibco) supplemented with 5% dialysed newborn calf serum and 2% minimal essential medium. G_1-phase cells were taken 2 h after release of the mitotic block induced by N_2O and *S*-phase cells 2 h after release of the second thymidine block.

(c) *Activated templates*

Activated calf thymus DNA was prepared as described by Baril *et al.* (1977). Calf thymus DNA was dissolved in 10 mM-Tris·HCl (pH 7·5), 2·5 mM-$MgCl_2$, 50 μg bovine serum albumin/ml and 0·5 mM-phenylmethylsulphonyl fluoride at a concentration of 1 mg/ml and incubated with DNase (0·5 μg/ml) for 10 min at 25°C before adding 0·1 mM-EGTA and heating at 65°C for 10 min.

Activated chromatin depleted of polymerase α activity was prepared as follows. Beads, containing 2×10^7 nuclei/ml agarose, were incubated at 37°C for 2 h and then at 0°C for 30 min in 10 mM-*N*-ethylmaleimide before they were subjected to electrophoresis (16 h, 1 V/cm, 4°C) in recirculating isotonic buffer supplemented with 25 mM-Tris·HCl (pH 8·0). After recovery, they were incubated with *Hae*III (500 units/ml; 37°C) or *Tth*HB8I (500 units/ml; 60°C) for 1 h.

(d) *Antibody binding*

The monoclonal antibody, SJK 132-20 (Bensch *et al.*, 1982; Miller *et al.*, 1985), directed against human DNA polymerase α (Pharmacia) was incubated (20 μg/ml) with encapsulated nuclei for 60 min at 0°C in the replication mixture before monitoring replication by the standard assay.

(e) *Analysis of polypeptides by gel electrophoresis*

When proteins were to be analysed 0·1 mM-phenylmethylsulphonyl fluoride was added to all buffers. Proteins in supernatants or column fractions were dialysed against 2×2 l of 10 mM-Tris·HCl (pH 7·5), and concentrated under vacuum. Proteins in beads were incubated with deoxyribonuclease I (50 μg/ml) and ribonuclease A (50 μg/ml) in 2·5 mM-$MgCl_2$ for 30 min at 37°C. Samples for electrophoresis were mixed with an equal volume of sample buffer (Laemmli, 1970), heated to 95°C for 5 min, and applied to a 12% acrylamide gel (Laemmli, 1970). Following electrophoresis (200 V/cm, 1·5 h) gels were stained with Coomassie blue, destained, dried and autoradiographs prepared using "pre-flashed" Fuji RX film and intensifying screens.

(f) *Analysis of protein complexes*

Protein complexes were analysed under non-denaturing conditions, by gel filtration in Sepharose 4B (Pharmacia) or electrophoresis in agarose gels. Supernatant fractions (up to 500 μl) containing soluble proteins were loaded on to a 40 ml column of Sepharose 4B equilibrated with isotonic buffer and 1 ml fractions collected. A mouse monoclonal immunoglobulin (Ig)M (approx. 900×10^3 M_r) and standard dyes (to determine the excluded (V_e) and total (V_t) column volumes) were eluted from the same column. Soluble protein complexes were also fractionated by electrophoresis (2 V/cm, 15 h) in 2% agarose gels containing isotonic buffer and "electro-blotted" onto nitrocellulose (Towbin *et al.*, 1979).

Nitrocellulose-bound protein complexes were visualized by autoradiography and further analysed by probing with an anti-human DNA polymerase α monoclonal antibody. After electro-blotting, nitrocellulose filters were incubated successively with phosphate-buffered saline, 0·2% Tween 20 and various additives: (1) 100 ml with 2% BSA, 20°C for 1 h; (2) 100 ml with no additive, 20°C, 3×10 min; (3) 10 ml with mouse monoclonal antibody (1 μg/ml) directed against human DNA polymerase α (SJK 132-20, Pharmacia), 20°C for 1 h; (4) 100 ml with 0·1% BSA, 20°C, 3×5 min and 3×20 min; (5) 10 ml with ^{125}I-labelled rabbit anti-mouse antibody (10^6 cts/min per ml; 50×10^6 cts/min per μg protein), 20°C for 1 h; and finally (6) 100 ml with 0·1% BSA, 20°C, 3×5 min and 3×20 min. Antibody binding was visualized by autoradiography.

(g) *Soluble DNA polymerase*

Following lysis of encapsulated cells proteins in the Triton supernatant were partially purified by gel filtration using 5 ml columns of Sephadex G100 and Sepharose 4B equilibrated with isotonic buffer.

3. Results

(a) *Preincubation generates a soluble activity*

We assay polymerase activity in encapsulated HeLa nuclei by incubating them in [^{32}P]dTTP and determining the incorporation of label into acid-insoluble material. Preincubation at 37°C reduces the rate of incorporation (Fig. 1(a)). Clearly the activity is labile in the isotonic buffer. A wide range of additives were tested (including bovine serum albumin, Ficoll, glycerol, sucrose, spermine, spermidine, the additives in the relication mixture, and trace amounts of Cu, Co, Ca, Zn, Mn, Mg) to see if any could stabilize the activity, but none had a marked effect (see Fig. 1 for the effect of Mg). The decline could be due to an irreversible loss of activity or to the dissociation of an activity from the template. Therefore encapsulated nuclei were incubated at 37°C and after various times the beads pelleted and any activity in the supernatant assayed using activated DNA as a template. Figure 2 shows that a soluble activity (i.e. α^{det}) appears in the supernatant as the insoluble activity (i.e. α^{nsk}) declines. We assume that α^{det} is derived from α^{nsk} by detachment from the nucleoskeleton, but it is difficult to prove formally that this is so (see Discussion).

(b) *Cutting the template stimulates activity*

The decline in α^{nsk} activity can only be seen if few breaks accumulate in the template during

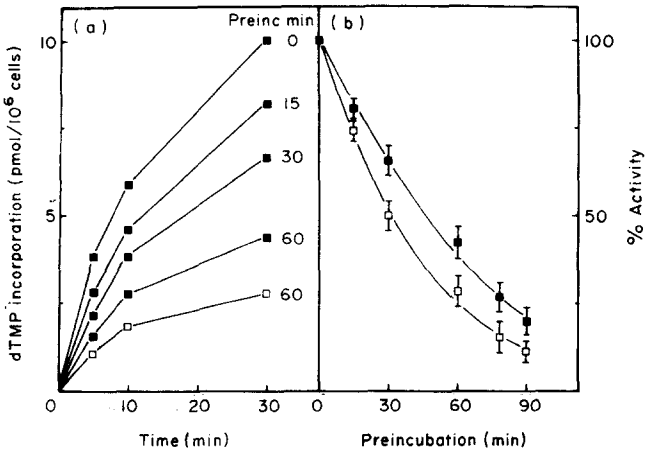

Figure 1. Pre-incubation at 37°C reduces the rate of DNA synthesis. (a) A typical experiment in which cells were labelled for 24 h with [^{3}H]thymidine, encapsulated and lysed. Samples were pre-incubated at 37°C in isotonic buffer with or without 2·5 mM-$MgCl_2$ for 0 to 60 min prior to measurement of the rate of incorporation of [^{32}P]dTMP into acid-insoluble material. Pre-labelling with ^{3}H enables us to check subsequently that each assay contained exactly equivalent numbers of nuclei. (b) Initial rates taken from a series of experiments similar to that illustrated in (a) are expressed as a percentage of the initial rate (± standard deviation) found without preincubation at 37°C, with (■) or without (□) 2·5 mM-$MgCl_2$.

preincubation, because they *stimulate* an activity initially present in the encapsulated nuclei. (See the accompanying paper (Jackson & Cook, 1986*b*) for evidence that few breaks accumulate.) This can be shown by growing cells in [^{3}H]thymidine to uniformly label their DNA, preincubating the resulting encapsulated nuclei with a nuclease to cut their DNA and finally measuring the rate of synthesis. Damage can be monitored by electro-eluting any chromatin fragments from the beads: the amount of chromatin (i.e. ^{3}H) remaining in beads reflects the degree of double-strand cutting. Figure 3 shows that pretreatment with *Hae*III or deoxyribonuclease both fragments chromatin and stimulates DNA synthesis. Pretreatment with *Eco*RI, an enzyme that cuts less frequently than *Hae*III, never stimulates synthesis more than 1·2 times (unpublished results; and Jackson & Cook, 1986). In general, the greater the fragmentation, the greater the stimulation, suggesting that the activity is stimulated by ends. However, high levels of deoxyribonuclease do not stimulate synthesis so markedly presumably because the template contains too many single-strand breaks for efficient synthesis. Like α^{nsk}, the activity seen after *Hae*III pretreatment is inhibited by aphidicolin (unpublished results).

Does the increased activity result from the provision of an "activated" template for α^{nsk} or for the α^{det}, which is inevitably formed during *Hae*III treatment? It is difficult to distinguish formally between these possibilities, but the experiment described in Figure 4 is most simply explained if it is α^{det} that is stimulated. Encapsulated nuclei were isolated from ^{3}H-labelled cells and the activity stimulated with *Hae*III. If polymerase that has dissociated from the nucleoskeleton and then subsequently associated with chromatin fragments generates the higher activity, electroelution should

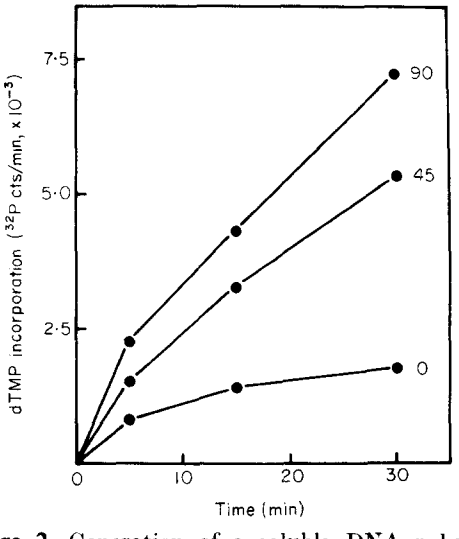

Figure 2. Generation of a soluble DNA polymerase activity. Cells were labelled with [^{3}H]thymidine, encapsulated and lysed; 500-μl samples were incubated at 37°C in isotonic buffer for 0, 45 or 90 min and then on ice for 60 min before pelleting. Activated calf thymus DNA (final conen 0·6 mg/ml) was added to the supernatant and the incorporation of [^{32}P]dTMP into acid-insoluble material measured.

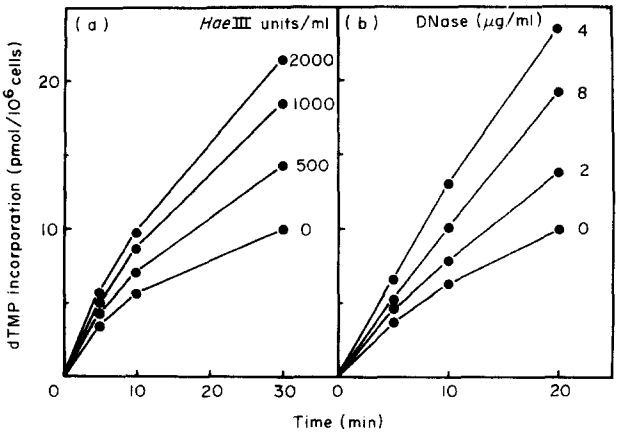

Figure 3. Activation of polymerase by cutting DNA in chromatin. Cells were labelled for 24 h with [^{3}H]thymidine, encapsulated and lysed. Nuclei in isotonic buffer containing 2·5 mM-$MgCl_2$ were pre-incubated with (a) *Hae*III (0 to 2000 units/ml) or (b) DNase I (0 to 8 μg/ml) for 60 min at 37°C. Finally, the rate of incorporation of [^{32}P]dTMP into acid-insoluble material was determined. The overall extent of cutting was estimated by subjecting samples to electrophoresis under isotonic conditions (following replication) and expressing the ^{3}H remaining in beads as a percentage of that present initially. As the amount of digestion increases, ^{3}H remaining fell to 76, 35 and 15% in (a) and 62, 50 and 18% in (b).

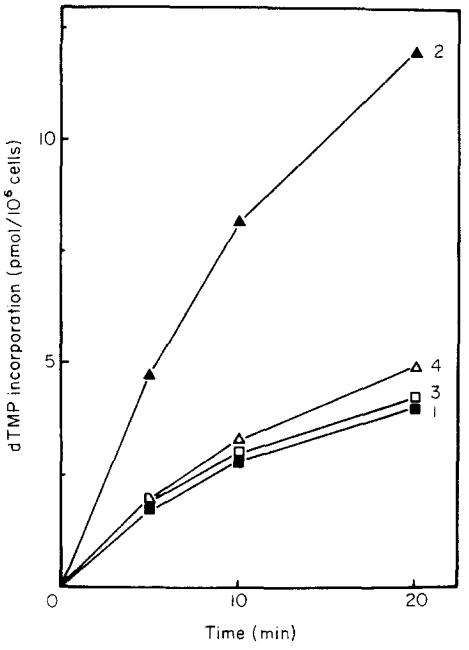

Figure 4. DNA polymerase associated with the nucleoskeleton. Cells were labelled for 24 h with [^{3}H]thymidine, encapsulated (2×10^7/ml) and lysed; 1-ml samples were incubated for 20 min at 37°C with or without *Hae*III (500 units/ml) in isotonic buffer containing 2·5 mM-$MgCl_2$. Samples were divided, half stored on ice and half subjected to electrophoresis in isotonic buffer containing 25 mM-Tris·HCl (pH 8·0) then washed once in 10 vol. isotonic buffer and the rate of incorporation of [^{32}P]dTMP into acid-insoluble material was determined. Curve 1, (■) untreated with *Hae*III, no electrophoresis; curve 2, (▲) treated with *Hae*III, no electrophoresis; curve 3, (□) untreated with *Hae*III, plus electrophoresis; curve 4, (△) treated with *Hae*III, plus electrophoresis. The sample used for curve 4 contained 36% of the ^{3}H present in those used for 1 to 3.

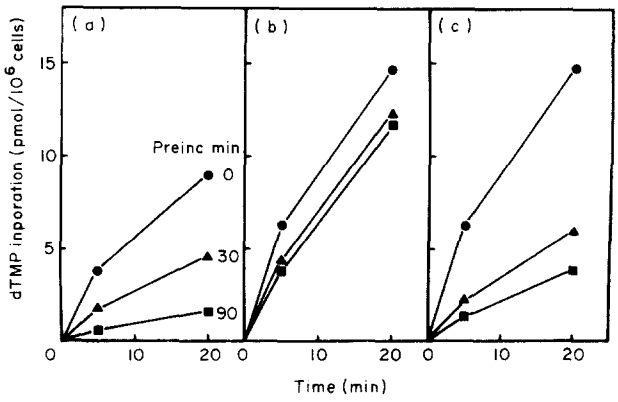

Figure 5. The soluble activity is stimulated by cutting with *Hae*III. Cells were labelled for 24 h with [^{3}H]thymidine, encapsulated and lysed. Nuclei in isotonic buffer were incubated for 0 (●), 30 (■) or 90 (▲) min at 37°C and then treated as follows: (a) 30 min on ice; (b) with *Hae*III (500 units/ml) at 37°C for 30 min; (c) washed twice with 5 vol. of isotonic buffer, then incubated at 37°C for 30 min with *Hae*III. Finally, the rate of incorporation was determined.

remove both it and the fragments from beads. This is what is found (see Fig. 4, cf. curves 2 and 4). Furthermore, the activity resisting electroelution is largely unaffected by *Hae*III (Fig. 4, cf. curves 3 and 4), implying that α^{nsk} cannot use a cut template.

In contrast, α^{det} *is* stimulated by *Hae*III cutting (Fig. 5). Encapsulated nuclei were preincubated for 30 or 90 minutes so that α^{nsk} activity declined (Fig. 5(a)) and α^{det} activity appeared (Fig. 5(b)). After cutting, the combined activities supported a high incorporation rate (Fig. 5(b)) but removal of α^{det} by washing reduced the rate (Fig. 5(c), compare curves for 30 and 90 min in (b) with those in (c)).

We next investigated the size of nascent DNA synthesized after cutting. Nascent DNA was synthesized *in vitro* using [^{32}P]dTTP and the cut template denatured and fractionated by electrophoresis (Fig. 6). Nascent DNA synthesized by α^{nsk} serves as a control for comparison (Fig. 6(a)). (See the accompanying paper for a detailed analysis.) Twenty-two per cent of the ^{32}P is in fragments <2000 nucleotides long (i.e. in Okazaki fragments and ligation intermediates) whilst the remainder has been ligated with longer molecules and is unable to enter the gel (Fig. 6(a)). Pretreatment with *Hae*III increased the incorporation 2·5 times and, after pelleting, 4% of the chromatin remained in the supernatant and contained small DNA fragments of <5000 nucleotides (Fig. 6(c), ^{3}H profile). The polymerase associated with this 4% was a further 2·5 times more active than the total contributing 10% of the synthesis, and yielding a range of ^{32}P-labelled fragments of <2000 nucleotides. Perhaps surprisingly for an activity that we believe initiates at ends, this distribution does not contain very short fragments a few nucleotides long or many end-labelled template fragments. Of course, no very long molecules are labelled because the template is so small. The pellet obtained after pretreatment with *Hae*III contained 96% of the chromatin and the synthesized fragments had sizes intermediate between those found with the unbroken and soluble chromatin templates (Fig. 6(b)).

(c) *Activated calf thymus DNA as a template*

The experiments described so far have all involved the endogenous template. Can the various activities use an added template? Addition of a weight of activated DNA equal to that in the endogenous intact chromatin has little effect (Table 1): clearly α^{nsk} prefers the native template. Addition of activated DNA to *Hae*III-treated chromatin also has little effect, presumably because all the α^{det} is bound to DNA fragments. This is true both of the high activity in *S*-phase nuclei and the residual activity in G_1 nuclei.

(d) *An activated chromatin template*

The previous experiments suggest that α^{det} might be best assayed using a fragmented chromatin

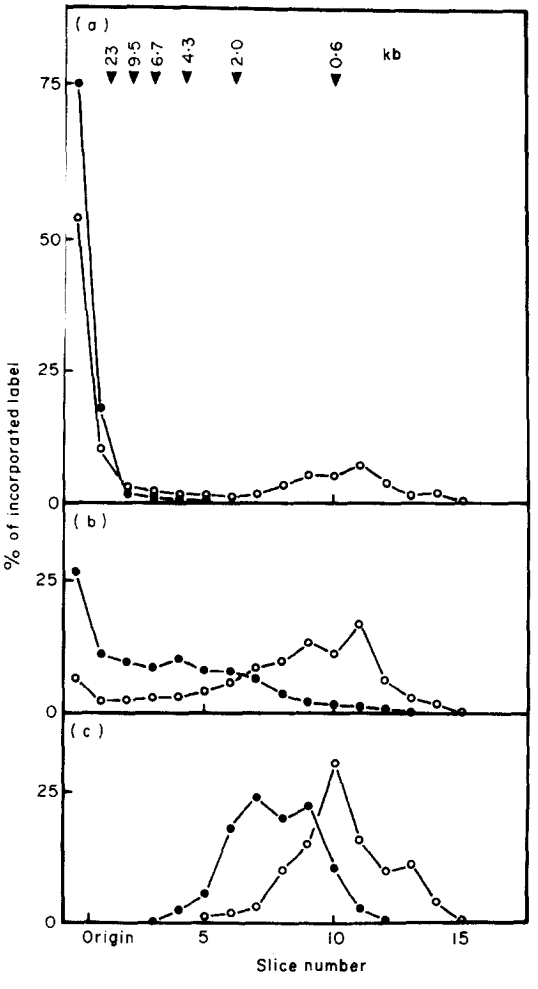

Figure 6. The size of nascent DNA synthesized with activated chromatin as the template. Cells were labelled for 24 h with [^{3}H]thymidine, encapsulated and lysed. Encapsulated nuclei were incubated with an equal volume of isotonic buffer containing 2·5 mM-$MgCl_2$, with or without *Hae*III (500 units/ml) for 30 min at 37°C. Samples were incubated on ice for 60 min to allow any soluble material to equilibrate, and then pelleted. The incorporation of [^{32}P]dTMP into acid-insoluble material by the pellet and supernatant was determined after 15 min and DNA from 25-μl samples purified, denatured, subjected to electrophoresis and acid-insoluble radioactivity in gel slices determined. The ^{3}H (●) and ^{32}P (○) recovered in each slice is expressed as a percentage of the total in each sample. The first point on each profile represents label recovered in beads and the second in the first gel slice. The positions of size-markers are indicated in kb. (a) Untreated with *Hae*III, pellet; this contained all ^{3}H and all ^{32}P. (b) Treated with *Hae*III, pellet; this contained 96% of the ^{3}H and 228% of the ^{32}P found in (a); (c) supernatant from (b), which contained 4% of the ^{3}H and 26% of the ^{32}P found in (a).

template. However, the endogenous activity in such chromatin might obscure the activity to be assayed. Therefore a suitable activated chromatin template was prepared as follows (see Materials and Methods for details). Encapsulated nuclei were depleted of insoluble activity by preincubation at 37°C for 2 h and any remaining activity inhibited by *N*-ethylmaleimide. Next, excess inhibitor and soluble protein were removed by electroelution before the chromatin was activated by treatment with *Hae*III. (In those cases where subsequent assays involve incubations with an added intact template, *Tth*HB8I, a restriction enzyme active at 65°C but not at 37°C, can be used instead of *Hae*III.) This activated chromatin has no polymerase α activity and is a suitable template for soluble enzymes (e.g. see Fig. 10).

(e) *The effects of various salt concentrations*

Increasing the concentration of KCl during replication progressively reduces the activity of α^{nsk}, but initially increases and then decreases the activity of α^{det} (Fig. 7). As a result, the salt concentration that we use routinely (i.e. 100 mM-KCl) is sub-optimal for α^{nsk} but optimal for α^{det}. The inhibition of both activities by 300 mM-KCl can be partly reversed by restoring the tonicity to normal prior to assay (Fig. 8). We find these salt effects very surprising and have no complete explanation for them, but include them because salt extractions are so commonly used when purifying polymerases.

(f) *Differential binding of an anti-polymerase antibody*

Korn and co-workers have characterized a range of monoclonal antibodies raised against purified polymerase α from KB cells (Bensch *et al.*, 1982; Miller *et al.*, 1985). Therefore, we tested whether our two activities were equally sensitive to inhibition by one of their antibodies, SJK 132-20. This antibody neutralizes polymerase α in permeabilized cells (Miller *et al.*, 1985). α^{nsk} proved to be completely resistant to levels of antibody known to inhibit the pure KB enzyme *in vitro* (Miller *et al.*, 1985), whereas the activity stimulated by *Hae*III was partly inhibited (Table 2). As we know that a range of monoclonal antibodies directed against various nuclear matrix proteins are able to penetrate beads and bind to nuclear elements (unpublished results), this failure to inhibit α^{nsk}, even at the high concentrations used in Table 2, is most probably due to masking of binding sites. (However, see Miller *et al.* (1985) for a cautionary note on the interpretation of studies like these using the same antibody.)

Whatever the precise reason for the failure to inhibit α^{nsk}, it is clear that the antibody can be used to distinguish the two activities.

(g) *The proteins solubilized on incubation at 37°C*

The polypeptides lost from encapsulated chromatin on incubation at 37°C were analysed by electrophoresis in gels containing sodium dodecyl sulphate (Fig. 9(a)). Cells were grown in [^{35}S]methionine, encapsulated, lysed and any

Table 1
Stimulation of DNA polymerase activity by added templates

Encapsulated nuclei	Activity (%)	Stimulation		
		+ Activated DNA	Pretreatment with *Hae*III	+ Activated DNA and pretreatment with *Hae*III
Unsynchronized	30–35	1–1·3×	2–3×	2–4×
G_1-phase	2–5	1–1·5×	4–6×	4–8×
S-phase	100	1–1·3×	1·5–2×	1·5–2·5×

Cells were labelled for 24 h with [^{3}H]thymidine, synchronized as appropriate, encapsulated and lysed. The incorporation of [^{32}P]dTMP into acid-insoluble material by equal numbers of encapsulated nuclei was determined after 30 min and expressed as a percentage of the value obtained with *S*-phase cells. In addition, the stimulation by added activated calf thymus DNA (100 μg/ml), or by activating the endogenous template by pretreatment with *Hae*III, or both, was determined. Values shown represent the range found in different experiments.

unattached protein was removed by electrophoresis. Next, beads were incubated in various buffers to permit dissociation. Those incubated on ice lose only 0·2% of their protein and serve as a control. After pelleting the beads, the supernatant was applied to a gel. Lanes 1 and 2 show the proteins of a control pellet and supernatant. Incubation at 37°C, whether in the isotonic buffer (lane 3) or the replication mixture (lane 4) selectively extracts about 8% of the protein. (Note that protein from different numbers of nuclei are loaded to aid comparison.) However, this loss does not result from a general disintegration of nuclei, since specific polypeptides are solubilized (Fig. 9, lanes 1 and 3 or 4). For example, no core histones are lost even though many small polypeptides are solubilized.

We next investigated whether these polypeptides dissociated from the nuclei or chromatin as part of a large complex (Fig. 10). After incubation at 37°C in isotonic buffer, the supernatant was applied to a column that fractionates proteins in the range 5×10^4 to 5×10^7 M_r. Labelled proteins eluted over the whole range, but polymerase (assayed using activated chromatin) eluted in two peaks, close to the position of elution of IgM (about 10^6 M_r). Assays using activated DNA gave a similar profile (unpublished results). Fractions from this column were pooled, concentrated and analysed by gel electrophoresis (Fig. 9, lanes 7 to 16). Encapsulated nuclei (lane 5) and material loaded on to the column

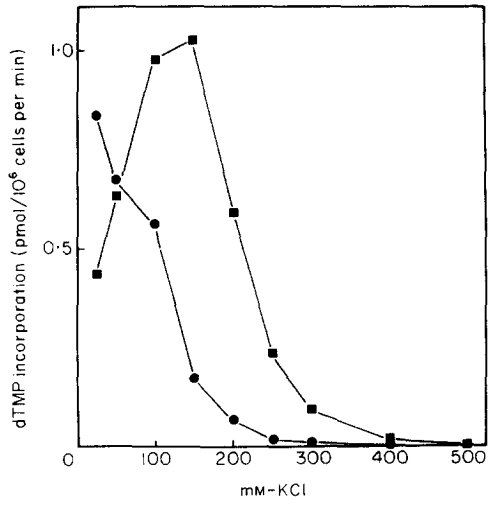

Figure 7. The effect of ionic conditions on different polymerase α populations. Cells were labelled for 24 h with [^{3}H]thymidine, encapsulated and lysed. One sample (●) was incubated on ice and a second (■) digested with *Hae*III (500 units/ml) at 37°C, for 2 h. Replication assays were performed on parts of each sample using the standard mixture with 25 to 500 mM-KCl. For each salt concentration, samples were taken at 0, 5 and 30 min and the initial rate of incorporation was determined.

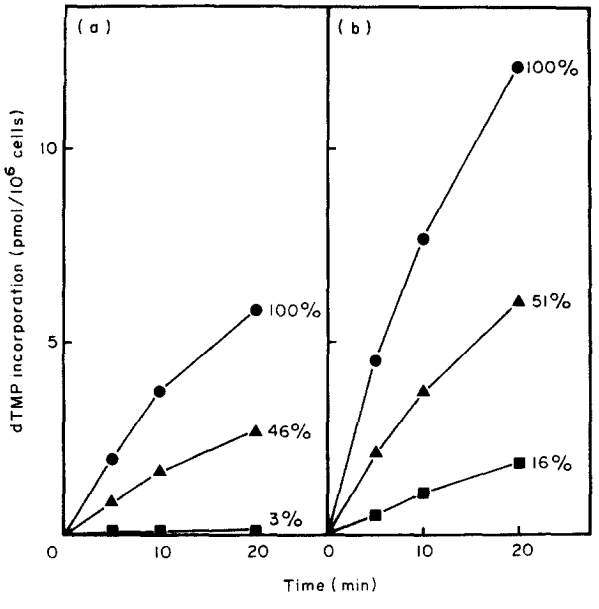

Figure 8. The effect of salt treatment on stimulated polymerase activity. Cells were labelled with [^{3}H]thymidine, encapsulated and lysed. Nuclei in isotonic buffer containing 2·5 mM-$MgCl_2$ were incubated for 30 min at 37°C (a) without or (b) with *Hae*III (500 units/ml). The rate of incorporation was measured after various treatments: (●) 15 min incubation on ice; (■) 0·3 M-KCl, 15 min incubation on ice; (▲) 0·3 M-KCl, 15 min incubation on ice then return to 0·1 M-KCl by dilution with isotonic buffer containing no KCl.

Table 2

Inhibition of replication by an antibody to DNA polymerase α

Sample	Treatment	Initial rate of incorporation (pmol/10^6 cells per min)	
		− Antibody	+ Antibody
1	Control	0·62	0·61
2	Preincubation, 37°C	0·19	0·20
3	*Hae*III treatment, 37°C	1·39	0·86

Cells were labelled for 24 h with [^{3}H]thymidine, encapsulated, lysed and: sample 1, stored on ice; sample 2, preincubated at 37°C for 60 min; sample 3, preincubated at 37°C for 60 min with *Hae*III (500 units/ml for 30 min). Next, 500-μl samples containing 3×10^6 nuclei were incubated ± 10 μg monoclonal antibody to DNA polymerase α on ice for 30 min. Finally, the rate of incorporation was determined.

(lane 6) are included for comparison. Fractions possessing most polymerase activity (i.e. lanes 9 and 13) contain numerous polypeptides that must have eluted as a large complex. Unfortunately, the profile is too complicated to permit identification of polypeptides associated with activity. However, it is clear that α^{det} is more complex than we initially imagined, being composed of at least two activities, with masses of about $0{\cdot}7 \times 10^6$ and $2{\cdot}5 \times 10^6$ M_r.

Electrophoretic analysis of supernatants containing α^{det} activity confirms this heterogeneity. A supernatant like that used for the Sepharose

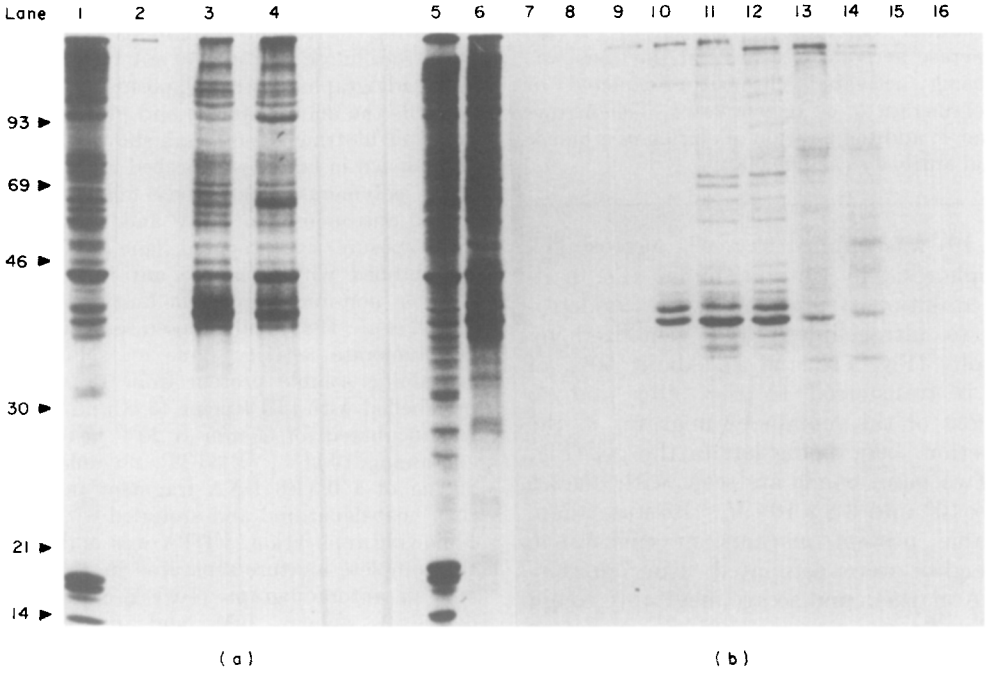

Figure 9. Analysis of soluble polypeptides in acrylamide gels. (a) Polypeptides extracted from encapsulated nuclei on incubation. Cells were labelled for 16 h with [^{35}S]methionine, encapsulated (5×10^7 cells/ml) and lysed. Beads were washed twice in 50 vol. isotonic buffer and subjected to electrophoresis in isotonic buffer containing 25 mM-Tris·HCl (pH 8·0) for 3 h (4 V/cm), to remove any unattached protein. Beads were recovered, washed once in 10 vol. isotonic buffer and samples incubated in isotonic buffer on ice, at 37°C or in the replication mixture at 37°C with no label added. Samples were incubated for 1 h, beads pelleted and proteins in pellet and supernatant fractions resolved by gel electrophoresis, and an autoradiograph prepared. Lane 1, incubated in isotonic buffer at 0°C, pellet; lane 2, incubated in isotonic buffer at 0°C, supernatant; lane 3, incubated in isotonic buffer at 37°C, supernatant; lane 4, incubated in replication mixture at 37°C, supernatant. Lane 1 contains protein from 10^5 encapsulated nuclei, and lanes 2 to 4 protein solubilized from 10^6 encapsulated nuclei. The % protein solubilized was 0·2, 7·5 and 8·0% in lanes 2 to 4, respectively. (b) Polypeptides in complexes released from encapsulated nuclei on incubation. Samples from the experiment described for Fig. 10 were subjected to electrophoresis and an autoradiograph prepared. Lane 5, polypeptides in encapsulated nuclei prior to incubation at 37°C; lane 6, supernatant loaded on column; lanes 7 to 16, pooled column fractions from Fig. 10. Column fractions 10 to 12 are in lane 7, fractions 13 to 15 in lane 8 etc.

The positions of migration of proteins of known sizes (in $M_r \times 10^{-3}$) are indicated on the left. Exposures were for 2 weeks.

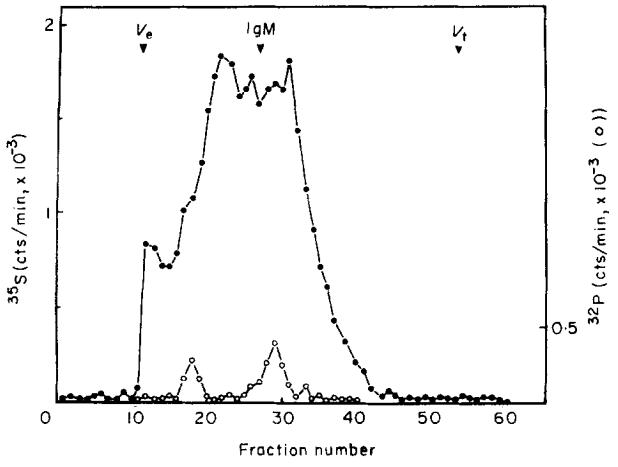

Figure 10. The size of soluble activities generated by incubation at 37°C. Cells were labelled for 24 h with [^{35}S]methionine, encapsulated (5×10^7 cells/ml), lysed and washed twice in 50 vol. isotonic buffer, before removing any unattached material by electrophoresis in isotonic buffer containing 25 mM-Tris·HCl (pH 8·0) for 3 h (4 V/cm). Next, beads were washed once in 10 vol. isotonic buffer and incubated at 37°C for 1 h to generate the soluble activity. Finally, beads were pelleted and proteins in the supernatant (500 μl containing $2{\cdot}5 \times 10^7$ cell-equivalents of soluble protein) fractionated on a Sepharose 4B column. The radioactivity due to ^{35}S (●) and the polymerase activity (○) in 1-ml fractions was determined (using activated chromatin depleted of endogenous polymerase α as a template). The arrows represent V_e and V_t and the position of elution of a mouse IgM monoclonal antibody (M_r 900,000).

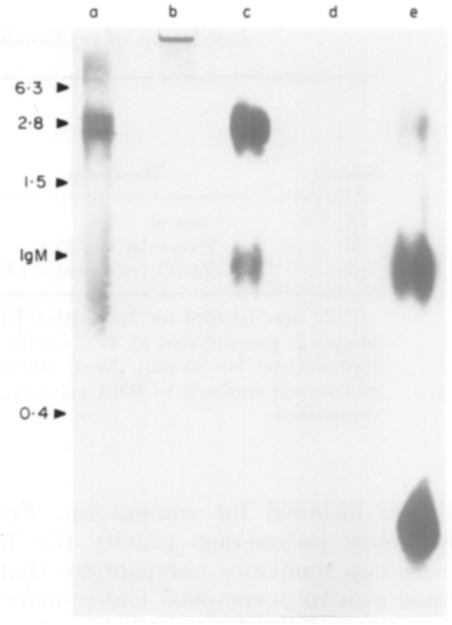

Figure 11. Analysis of soluble polymerase complexes. Immunoblotting (lanes a to d): 10^6 cell equivalents (20 μl) of soluble protein labelled with ^{35}S and prepared as described in the legend to Fig. 10 were separated by electrophoresis in 2% agarose containing isotonic buffer. Following electrophoresis proteins were electro-blotted on to nitrocellulose (6 h at 150 mA in 0·5 × isotonic buffer). Autoradiographs (2-week exposure) of protein migrating towards the anode (lane a) and protein remaining in the gel after blotting (lane b) are shown. A second filter like that shown in lane a was probed with mouse anti-human DNA polymerase α antibody followed by ^{125}I-labelled rabbit anti-mouse antibody and an autoradiograph (3-day exposure) was prepared (lane c). The control (lane d) was probed with a mouse anti-human IgM against a suitable non-nuclear protein (antibody to Ca1 antigen; Ashall *et al.*, 1982) and exposed for 2 weeks.

Polymerase activity (lane e): soluble extract (20 μl containing soluble protein from 10^6 nuclei) from cells prelabelled with [^{3}H]leucine (5 μCi/ml, 50 Ci/mmol, 18 h) was incubated for 60 min at 20°C in replication mixture containing 10 μCi [^{32}P]dTTP, no unlabelled dTTP and 250 ng of a 0·3 kb DNA fragment purified from a gel, then heat-denatured and annealed with 0·1 unit p(dN)$_6$. Following replication, EDTA was added to 2·5 mM and the complete mixture separated in 2% agarose, blotted, and an autoradiograph (1-week exposure) prepared as described above. IgM and double-stranded DNA fragments provide markers ($M_r \times 10^{-6}$).

column (Fig. 10) was applied to a 2% agarose gel. After electrophoresis in isotonic buffer (i.e. non-denaturing conditions) proteins were "electro-blotted" on to nitrocellulose and visualized by autoradiography (Fig. 11, lane a). About 50% of the protein is transferred to the filter and so visualized; most of the remainder migrates in the opposite direction, little being left in the gel (Fig. 11, lane b). Two main bands are seen, with masses of about $2{\cdot}6 \times 10^6$ and $3{\cdot}2 \times 10^6$ M_r. (Because there are no suitable protein markers in this range molecular weights were estimated using double-stranded DNA markers and so are inevitably rough estimates.) These two protein complexes represent about 5% of the protein originally applied to the gel.

Polymerase α in these gels was detected using antibodies and by direct assay. After blotting, filters were incubated with two different antibodies, one a monoclonal directed against polymerase α and the other (the control) a monoclonal directed against human Ca1 protein (Ashall *et al.*, 1982). Binding was detected using a second ^{125}I-labelled anti-mouse antibody and autoradiography (Fig. 11, lanes c and d). Two populations of DNA polymerase α were identified, a major one in the region of the two predominant protein complexes and a minor one close to the position of migration of an undenatured IgM. These same regions contained synthetic activity (Fig. 11, lane e). Prior to electrophoresis, the supernatant was incubated in replication mixture containing [^{32}P]dTTP and single-stranded DNA fragments to which had been annealed primers, six nucleotides long. After addition of EDTA, protein–template complexes were subjected to electrophoresis, electro-blotted and visualized by autoradiography (Fig. 11, lane e). Three regions of label are seen, the two identified by antibody binding, which presumably contain complexes of nascent DNA and protein, and a third, intensely labelled region corresponding to the position of the free template. Although it remains possible that this assay detects DNA-binding

Table 3
A soluble polymerase that cannot use intact templates

		Supernatant			
		None	U	G_1	*S*
Pellet, intact chromatin	U	33	33	33	36
Pellet, intact chromatin	G_1	3	3	3	3
Pellet, intact chromatin	*S*	100	100	100	110
Pellet, activated chromatin	U	100	365	365	497
Pellet, activated chromatin	G_1	20	250	249	325
Pellet, activated chromatin	*S*	200	675	635	735

Cells were labelled with [^{3}H]thymidine and equal numbers of unsynchronized (U), G_1 or *S*-phase cells encapsulated and lysed. Soluble polymerase obtained after lysis was freed of EDTA and Triton X-100 and 50 μl of each (containing 10^5 cell-equivalents) incubated for 1 h on ice with 100 μl containing $6{\cdot}6 \times 10^5$ encapsulated nuclei, untreated ("intact chromatin") or pre-treated with *Hae*III ("activated chromatin"). Incorporation of [^{32}P]dTMP into acid-insoluble material was determined after 30 min. Values give activity expressed as a percentage of that present in *S*-phase nuclei incubated without added extract.

proteins rather than polymerase, the coincidental labelling by antibody suggests the presence of active DNA polymerase complexes.

(h) *A soluble activity (α^{sol})*

All the experiments described so far have involved an activity (or its derivatives) that pelleted with beads on lysing cells. However, an additional aphidicolin-sensitive activity fails to pellet. This is probably the "cytoplasmic" polymerase that has been widely studied by others using activated DNA as a template (Kornberg, 1974). This soluble activity (α^{sol}) cannot replicate chromatin containing intact DNA, but can replicate activated chromatin (Table 3). Cells from different stages of the cell cycle were encapsulated, lysed and pelleted. The pellets provide both an intact template and α^{nsk} activity, which reflects the phase of the cycle from which the chromatin was derived. (See accompanying paper for a detailed analysis of activity during the cell cycle.) The supernatants provide a source of α^{sol}, which is assayed after mixing the various supernatants and pellets. The activity is expressed as a percentage of that found in *S*-phase pellets without added supernatant. Addition of the supernatants hardly, if at all, stimulates synthesis above that due to α^{nsk} already in the pellet. These supernatants *do* possess an activity since they stimulate synthesis in activated chromatin (Table 3) or calf thymus DNA (unpublished results). However, there is little quantitative variation in α^{sol} activity throughout the cell cycle: *S*-phase cells having approximately 1·5 times the activity of their G_1-phase counterparts. Comparing the activity of α^{det} and α^{sol} (Table 3) implies that only 5 to 10% of the DNA polymerase α within *S*-phase cells is present as α^{nsk}.

Table 4
The properties of three different polymerase activities

Property	Activity		
	α^{sol}	α^{nsk}	α^{det}
Tight association with nucleoskeleton	−	+	−
Activity on native template	−	+	−
Activity on activated template	+	−	+
Cell-cycle dependence	−	+	+

4. Discussion

(a) *Three different activities*

We have described at least three different aphidicolin-sensitive polymerase activities, which have been distinguished using three main criteria: the tightness of their association with encapsulated chromatin (e.g. whether they resist electroelution from beads), their response to different ionic conditions (Fig. 7) and their template preferences (Table 4). One activity (α^{nsk}) has been studied in greater detail in the accompanying paper and elsewhere (Jackson & Cook, 1986*a*). It synthesizes DNA *in vitro* at 85% of the rate *in vivo*, it is only found in *S*-phase nuclei and requires unbroken DNA in the form of chromatin as a template. Presumably active polymerase, tightly bound to some nuclear substructure, was halted by lysis as it was replicating its natural template: when the conditions necessary for replication are restored, this activity is seen as α^{nsk}.

It is difficult to prove definitely how the other activities are related to α^{nsk} for at least two reasons. First, no single template serves them all, so that their relative activities cannot be compared directly. Second, since binding sites in α^{nsk} may well be masked (Table 2) their relative abundance cannot be measured using the monoclonal antibody. Despite these reservations, it seems likely that α^{nsk} detaches from the nucleoskeleton on incubation at 37°C to give α^{det}, which must be assayed using a

new template and primer. Thus any assay of α^{nsk} will inevitably be obscured by some contribution from α^{det} generated during the assay. However, this contribution can be minimized by denying α^{det} its preferred template (i.e. chromatin containing broken DNA).

With these reservations in mind, we can make a very rough estimate of the relative proportions of α^{nsk} and α^{sol} in the cell, if we assume that all α^{nsk} is converted to α^{det} on incubation for two hours at 37°C. Then this α^{det} (and so α^{nsk}) has about 1/20 the activity of α^{sol} from the same number of cells, when both are assayed using a *Hae*III-cut template (Table 3).

The major activity studied by many others, which is assayed using broken templates, varies in amount throughout the cell cycle (Thömmes *et al.*, 1986) and leaches out of the nucleus when a cell is lysed (for reviews, see Kornberg, 1974; De Pamphilis & Wassarman, 1980; Thömmes *et al.*, 1986). Our α^{sol} resembles this activity. Whether it truly is a soluble nuclear enzyme or whether it dissociates from a larger structure on lysis is uncertain. Whichever view is correct, it is attractive to suppose that α^{sol} provides an inactive pool of polymerase, awaiting attachment or stabilization at the nucleoskeleton prior to initiation of DNA synthesis. Our rough estimate indicates that this pool of α^{sol} is 20 times larger than that of α^{nsk}.

(b) *Soluble DNA polymerase activities*

Gel chromatography and electrophoresis show α^{det} to be composed of at least two activities. One, of about 750×10^3 M_r, is probably a core polymerase complex and the other, composed of two protein complexes of about 3×10^6 M_r (Figs 10, 11 and unpublished results), may be related to the "replitase" described by Noguchi *et al.* (1983). α^{sol} is also composed of a number of related complexes (unpublished results). We imagine that the relative constitution of these complexes varies as a cell progresses through the cell cycle and determines whether or not they become activated by association with the nucleoskeleton.

(c) *A system for replication* in vitro

This study has mainly concerned activities that must be aberrant, since they only replicate broken templates. We hope that their distinction from the more authentic activity, α^{nsk}, will provide a basis for further studies using encapsulated chromatin. This preparation, whilst having the disadvantage of a complex structure, nevertheless has the great advantages of its unbroken template, an efficient activity and (perhaps most importantly) the nucleoskeleton thought necessary to order a process as complicated as replication (see Ariga & Sugano, 1983; Li & Kelly, 1984; Jazwinski & Edelman, 1985; Wobbe *et al.*, 1985, for some studies using simpler systems).

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