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A Study of the Template Properties of Chromatin for DNA Polymerase I and of the Effects of Ionising Radiation

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The template activity of rat thymus chromatin for *Escherichia coli* DNA polymerase I has been found to amount to only 6% of that of pure DNA. Assays have been carried out on chromatin in 0.05 M NaCl, on a chromatin · polylysine complex, on the soluble and insoluble fractions of chromatin in the assay system and on DNA prepared from chromatin. The results suggest that the low activity is due only to a small extent to the insolubility of the chromatin in the assay system. Instead, it is proposed that initiation is restricted to certain "accessible" DNA zones in chromatin.

After γ-irradiation of the chromatin, the template activity increases with increasing dose up to a certain level and then decreases. It is therefore proposed that both active and inactive binding sites for DNA polymerase are formed on irradiation. Assays on a chromatin · polylysine complex indicate that the new initiation sites occur in the "accessible" zones.

Chromatin has been shown to be a very poor template for DNA polymerase I [1–5]. The reason for the low template activity is uncertain although it had previously been suggested that it was due to the low solubility of chromatin in the assay system [1,3,4]. In order to distinguish between several possible explanations, we have measured the template activity of the whole chromatin suspension, of the fractions soluble and insoluble in the DNA polymerase I assay system under various conditions, and on the constituent DNA. We conclude that the insolubility of chromatin in the assay system is not the major factor responsible for its low activity. Instead, from studies with added polylysine, we propose that there are only a limited number of initiation sites of DNA synthesis and that these occur within certain zones of "accessible" DNA-phosphates in the chromatin.

The template activity of chromatin can be increased by exonuclease III [6] and by specific polyanions [7–9]. In the present study we have found that chromatin is activated also by ionising radiation and we propose that new initiation sites are produced in the accessible zones.

MATERIALS AND METHODS

Materials

DNA-dependent DNA polymerase I was prepared from *Escherichia coli* B (obtained as a frozen cell paste from M.R.E., Porton) by a slight modification of published procedures [10,11]. The enzyme used was the phosphocellulose fraction that had been further purified by ultra-centrifugation in a glycerol gradient. The four deoxyribonucleoside triphosphates and high-molecular-weight calf thymus DNA were purchased from Sigma London Chemical Co., Ltd. [^{3}H]dTTP was purchased from the Radiochemical Centre (Amersham) and poly[d(A-T)] was prepared by the method of Schachman *et al.* [12]. Rat thymus chromatin was prepared as described previously [13]. The final gel product contained about 37% nucleic acid, 49% histone and 14% non-histone protein. The gel was diluted in distilled water to a concentration of 0.02% DNA. Poly-L-lysine hydrochloride, mol. wt 14000, was obtained from Miles-Seravac Ltd (Maidenhead, Berks.).

Estimation of DNA and Protein

DNA concentrations were measured from absorbance at 260 nm, taking $A^{1\%}_{1\,cm}$ as 210. Protein concentrations were measured by a microbiuret method [14] or by the method of Lowry *et al.* [15].

Assay System

The standard assay (total volume 0.3 ml) contained 10 nmol of each of the four triphosphates (the dTTP carried a tritium label of 1.3×10^6 counts/min), 0.15 units DNA polymerase I, 0.07 M Tris-HCl pH 7.4, 7 mM $MgCl_2$, and 1 mM mercaptoethanol.

Enzyme. E. coli DNA polymerase I (EC 2.7.7.7).

(These conditions are optimal for DNA but not necessarily for chromatin.) Varying amounts of chromatin were added before incubation at 38 °C for 30 min, unless otherwise stated. The radioactivity that had been incorporated into acid-insoluble material was determined by the addition of 0.2 ml of an ice-cold mixture of 80% (w/v) trichloroacetic acid, saturated $Na_2HPO_4 \cdot 12\ H_2O$, saturated $Na_4P_2O_7 \cdot 10\ H_2O$ (1:1:1, v/v/v). After filtration through a nitrocellulose filter (Sartorius 0.45 μm), the assay tube and the filter were washed with several portions of ice-cold 5% (w/v) trichloroacetic acid. After drying, the filter was placed in a toluene liuidfluor containing 0.4% PPO and 0.01% POPOP and the radioactivity was measured. 130 counts/min in the precipitated material corresponded to 1 pmol dTTP incorporated into newly synthesised DNA.

Preparation of the Chromatin · Polylysine Complex

Chromatin and polylysine were mixed at concentrations such that the accessible DNA phosphates and the lysyl residues were equivalent [16]; the resulting complex was insoluble. To check that no excess polylysine was present, a sample was tested for absence of free lysyl residues by a method developed for estimating polylysine [16,17].

Separation of Soluble and Insoluble Fractions of Chromatin in the Assay System

The chromatin was mixed with NaCl and/or assay mixture and was kept at 0 °C for at least 30 min before centrifuging at $3000 \times g$ for 15 min. The supernatant and the precipitate were kept and assayed separately. (The precipitate was assayed by resuspending in fresh assay mixture in the absence of enzyme.) In order to measure the solubility of chromatin in the assay mixture, the triphosphates, radioactive material and enzyme were omitted and the supernatant was analysed for DNA and protein.

Preparation of DNA from Chromatin

The chromatin solution was made 2 M in NaCl and 1.0 ml was layered on top of 4.5 ml of 5% sucrose in 2 M NaCl. After spinning for 18 h at 39000 rev./min in a Spinco SW39 rotor at 4 °C, the supernatant was removed and the pellet was dissolved in 0.01 M NaCl. The pelleted material was found to contain about 95% of the original DNA. Protein analysis showed the supernatant to contain at least 95% of the original protein.

Irradiation of Chromatin Solutions

The chromatin was irradiated at a concentration of 0.02% in distilled water (unless otherwise stated) in the presence of air. The source was ^{137}Cs with a dose rate of 8.4 rads s^{-1}.

Table 1. *Template activity and solubility of chromatin under various conditions*

Assay template	0.05 M NaCl added	dTTP incorporated	Solubility in assay mixture *cf.* original DNA
		pmol	%
1. Chromatin	—	4.3[a]	11
2. Chromatin	+	2.6 (3.3)[b]	5
3. Chromatin · polylysine[c]	—	<0.5	<2
4. Chromatin · polylysine[c]	+	<0.5	<2
5. Poly[d(A-T)]	—	360	
6. Poly[d(A-T)]	+	292	
7. Calf thymus DNA	—	70.4	
8. Calf thymus DNA	+	55.5	

[a] The figure quoted is the average of several independent experiments. The range of values was 3.7 to 4.9 pmol.

[b] The figure in brackets denotes dTTP incorporated after correction for the effect of 0.05 M NaCl on the DNA polymerase I: assays 5 and 6, and 7 and 8 show that the enzyme retains 80% of its activity in the presence of 0.05 M NaCl.

[c] Chromatin polylysine denotes the complex formed between chromatin and polylysine when the latter is added at a concentration sufficient to neutralise all the accessible DNA phosphates.

RESULTS AND DISCUSSION

Establishment of Conditions for Assaying Chromatin

The template activity of chromatin was found to increase linearly with increasing DNA concentration in the assay system up to at least 20 μg/0.3 ml. The incorporation increased linearly with time up to 30 min and then more slowly up to at least 120 min. Subsequent assays were carried out at a DNA concentration of 10 μg/0.3 ml assay mixture, incubating for 30 min.

Template Activity of Chromatin

Table 1 shows the template activity of chromatin, DNA, and poly[d(A-T)] under various conditions. It can be seen that the incorporation with chromatin as template is only about 6% of that with DNA (assays 1 and 7). There are several possible explanations for this low activity: (a) the low solubility of chromatin in the assay system (a fibrous suspension was observed and removal of this by centrifugation showed that the supernatant contained only 11% of the original DNA); (b) a low accessibility of the DNA in chromatin to DNA polymerase because of the coverage by protein; (c) the presence of fewer breaks and hence initiation sites in chromatin compared with DNA, due to the mild preparative procedure used for the former; (d) a lower rate of DNA synthesis than on a DNA template, per enzyme molecule.

Assays in the Presence of 0.05 M Sodium Chloride and on a Chromatin · Polylysine Complex

To study the dependence of activity on the solubility of the chromatin, the assay system was made 0.05 M in NaCl, at which concentrations less than 5% of the chromatin is soluble (Table 1, assay 2) Measurements were made also using DNA and poly-[d(A-T)] as template in the above assay system so as to correct for the effect of the NaCl on the activity of the DNA polymerase. Table 1 shows that the NaCl reduces the activity of the DNA polymerase by 20% (assays 5 and 6, 7 and 8). For chromatin the incorporation decreases to about 77% and the solubility to about 50% of the corresponding values in the absence of NaCl (Table 1, assays 1 and 2).

To test the second possibility, assays were made on chromatin · polylysine complexes. Previous work had shown that about 40—45% of the DNA phosphates in chromatin are accessible to polylysine [16] while enzymic studies had indicated that most or all of the DNA is associated with protein [18]. It was therefore proposed that the fairly extensive zones of accessible DNA phosphates were associated possibly with non-basic stretches of histones. In the present study, sufficient polylysine was added to chromatin to neutralise these accessible DNA zones and assays were carried out on the resulting suspensions. It was found that both the template activity and the solubility of the chromatin · polylysine complexes in the assay mix were virtually zero (Table 1, assays 3 and 4).

Assays on the Soluble and Insoluble Fractions of Chromatin

From the above results it seemed at first that insolubility might indeed be the main cause of the low template activity of chromatin. However, separation and direct assay of that fraction of the chromatin which was soluble in the normal assay mixture showed that although its activity per unit weight of DNA was about 1½ times that of the original chromatin (but only 1/10 that of DNA), the total activity was less than 20% of that of the original suspension (see Table 2). Correspondingly, the activity of the insoluble fraction was about 80% of that of the original chromatin. To test that this difference was not due to preferential binding of the DNA polymerase to the insoluble chromatin, poly-[d(A-T)] was added to the superntanat; the incorporation observed was similar to that of a normal assay with poly[d(A-T)] showing that practically all of the enzyme was available in the supernatant. When poly[d(A-T)] was added to the resuspended pellet, little incorporation was observed. Also, addition of DNA polymerase to both soluble and insoluble fractions caused no significant increase in incorporation. The soluble fraction was found to have a ratio of DNA to protein similar to that of the original chromatin.

Table 2. *Template activity of the total suspension and of the soluble and insoluble fractions of chromatin under various conditions*

Assay	dTTP incorporated by			Solubility in assay mixture *cf.* original DNA
	Total suspension	Soluble fraction	Insoluble fraction	
	pmol	pmol	pmol	%
Normal assay	4.2	0.7	3.3	11
After addition of poly[d(A-T)] to each fraction		349	5.7	
After addition of more DNA polymerase to each fraction		0.8	3.5	
Normal assay in presence of 0.05 M NaCl	2.3 (2.8)[a]	<0.2	—	5

[a] The figure in brackets denotes dTTP incorporated after correction for the effect of 0.05 M NaCl on the DNA polymerase I.

Measurements were made also on the fraction soluble in the assay mixture in 0.05 M NaCl. The activity was found to be almost zero in contrast to that of the complete chromatin suspension in this assay mixture.

These results indicate that most of the template activity resides in the fraction of the chromatin that is insoluble in the assay mixture, *i.e.* the low activity of chromatin in the normal assay mixture is due only to a small extent to its insolubility. This conclusion is supported by the finding (see below) that chromatin can be activated 10-fold by γ-irradiation without any change in its solubility in the assay mixture. It could be argued that if the chromatin · polylysine complex and the chromatin in 0.05 M NaCl were more highly aggregated than chromatin in the normal assay mix, this could account for their low activity. However, on irradiation of chromatin in 0.05 M NaCl, the activity increases to double the value for unirradiated chromatin in assay mix alone. (The activation is lower than for chromatin irradiated in water presumably because of the protective effect of the NaCl.) The irradiation does not increase the solubility, nor apparently, the state of aggregation. Therefore the complete inactivity of the chromatin · polylysine is unlikely to be due merely to its insolubility or state of aggregation. Instead one can infer that the polylysine blocks sites that would otherwise be accessible to the DNA polymerase and therefore that in chromatin, initiation normally occurs at sites within zones of accessible DNA phosphates.

Table 3. *Template activity of DNA prepared from control and irradiated chromatin*

Assay template	dTTP incorporated
	pmol
1. Control chromatin	4.2
2. DNA prepared from control chromatin	64.8
3. Calf thymus DNA	69.5
4. Calf thymus DNA treated as in 2	67.5
5. Irradiated chromatin	43.4
6. DNA prepared from irradiated chromatin	65.4

Assays on the DNA Component of Chromatin

To find whether potential initiation sites are present but are inaccessible in other regions of the chromatin, DNA was prepared directly from the chromatin. To check that the preparative procedure did not activate the DNA, pure DNA was treated in a similar way. Table 3 shows firstly that the template activity of pure DNA is unaffected by the procedure and secondly that the activity of the DNA prepared from chromatin is similar to that of the pure DNA and very much greater than that of the original chromatin. This and the results with polylysine indicate that in chromatin many initiation sites are in fact present but are inaccessible to the DNA polymerase.

Effect of Ionising Radiation on the Template Activity of Chromatin

After irradiation of the chromatin, the template activity initially increases linearly with dose, the slope being dependent on the amount of chromatin added to the assay system (Fig. 1). At higher doses the activity of the chromatin decreases, the dose required for maximum activity depending upon the chromatin concentration in the assay system. Once the maximum activity has been reached, all values, regardless of the chromatin concentration lie on the same curve. Over this region, a plot of the reciprocal of the activity against dose is linear (Fig. 2).

A similar increase in template activity after irradiation followed by a decrease at higher doses occurs with DNA [19] and has been explained by the formation of active and inactive enzyme binding sites. The increase in template activity is due to the formation of new active binding sites on the DNA; once sufficient binding sites have been formed to saturate the enzyme, the radiation-produced inactive sites cause a decrease in the template activity. Presumably this explanation applies also to chromatin.

For DNA it has been shown that a plot of rate of DNA synthesis, R, against dose D, is linear. The ratio of slope to intercept on the ordinate gives the relative increase in active binding sites produced per unit dose, p [19]. Fig. 1 gives values of p for chromatin of 0.41, 0.51, and 0.53 (average 0.48) per krad for 10, 7 and 4 μg chromatin DNA per assay respectively. The variation in values of p probably results from the difficulty in measuring accurately the low values of R for unirradiated chromatin.

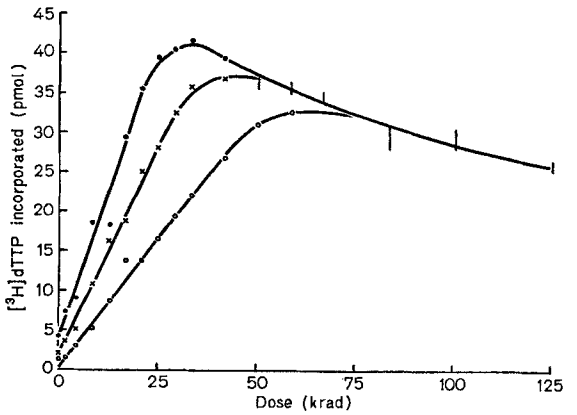

Fig. 1. *The effect of varying dose and chromatin concentration in the assay system on the template activity of chromatin.* (O) 4 μg chromatin per assay (0.3 ml); (×) 7 μg chromatin per assay; (●) 10 μg chromatin per assay. The vertical lines indicate the range of values observed for various concentrations of chromatin in the assay system

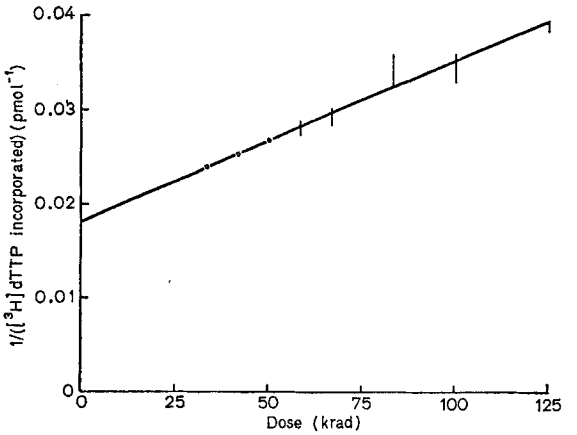

Fig. 2. *The reciprocal of template activity of chromatin plotted against dose.* The vertical lines indicate the range of values observed for various concentrations of chromatin in the assay system

Once the maximum rate of DNA synthesis has been reached, it has been shown for DNA that a plot of the reciprocal of the rate of DNA synthesis, $1/R$, against dose, D, is linear. The ratio of slope to intercept on the ordinate gives the relative increase in inactive binding sites produced per unit dose, q. Fig. 2 shows this to be 0.009 per krad for chromatin.

From the values of p and q for chromatin (0.48 and 0.009 per krad, respectively) we conclude that the majority of the radiation-produced enzyme-

Table 4. *Template activity of the total suspension and soluble fraction of chromatin under various conditions*
All assays contained chromatin, except 3 and 4 which had chromatin · polylysine. NaCl, where indicated, was 0.05 M and irradiation, where indicated was with a dose of 29.4 krad

Assay	0.05 M NaCl	Irradiated	dTTP incorporated by		Solubility in assay system *cf.* original DNA
			Total suspension	Soluble fraction	
			pmol	pmol	%
1.	−	−	4.1	0.7	11
2.	−	+	43.2	7.7	11
3. (Chromatin · polylysine)	−	−	<0.5		<2
4. (Chromatin · polylysine)	−	+	<0.5		<2
5.	+	−	2.3 (2.9)[a]	<0.2	5
6.	+	+[b]	9.8 (12.3)[a]	1.4 (1.8)[a]	5
7.	+	+	17.8 (22.0)[a]	5.0 (6.3)[a]	9

[a] The figures in brackets denote dTTP incorporated after correction for the effect of 0.05 M NaCl on DNA polymerase I. (The NaCl reduces the activity of the polymerase by 20%).
[b] This sample was irradiated as a suspension in 0.15 M NaCl.

binding sites in chromatin are active ($\approx 98\%$). However, in the case of DNA, only about 30% of the radiation-produced binding sites are active in DNA synthesis, p and q being 0.024 and 0.050 per krad, respectively.

The reason for the high proportion of active binding sites produced on irradiation of chromatin is uncertain. It is unlikely to be due to an unmasking of potentially active binding sites in the DNA which were previously covered by protein and inaccessible to DNA polymerase since there is little dissociation of chromatin on irradiation [20]. It could possibly arise from the interaction of the protein with the DNA in zones of accessible DNA phosphates.

From Fig. 2, by extrapolation to zero dose, it is possible to obtain the rate of DNA synthesis with a chromatin template under conditions of enzyme saturation (*i.e.* an excess of enzyme binding sites over enzyme molecules). This is found to be 55 pmol/30 min, and (taking into account the lower rate of synthesis of the insoluble fraction compared to the soluble fraction) compares favourably with the rate of synthesis with DNA as template. Thus the rate of DNA synthesis per enzyme molecule is very similar in chromatin and DNA.

Assays on an Irradiated Chromatin · Polylysine Complex and on the Soluble Fraction of the Irradiation Chromatin

Two questions arose from these results: (a) To what extent does the presence of protein and its arrangement on the DNA in chromatin affect the radiation damage in the DNA and (b) are most of the new binding sites produced in that fraction of the chromatin which is potentially soluble in the assay system? To answer the first question, assays were made on an irradiated chromatin · polylysine complex, and for comparison, on an irradiated suspension of chromatin insolubilised in 0.15 M NaCl. Table 4 shows that the activity of the irradiated chromtain · polylysine complex is zero (assays 3 and 4) in contrast to that of the irradiated chromatin suspension in salt (assays 5 and 6).

To answer the second question, the activity of the fraction of irradiated chromatin soluble in the assay system was measured. (Dose below 30 krad did not increase the solubility over the control value.) Assays were made also on the soluble fraction of the chromatin irradiated in suspension in 0.05 M NaCl. Table 4 (assays 2 and 6) shows that values of the activity of the soluble fractions were respectively 18% and 14% of that of the original suspensions, *i.e.* more than 80% of the activity occurred in the part of the chromatin that is insoluble in the assay system. Thus one can infer that the absence of any activation of the chromatin · polylysine complex on irradiation is not attributable to the insolubility of the complex.

The activation of chromatin when irradiated in NaCl is lower than when irradiated in water, values being respectively 4 times (Table 4, assays 6 and 7) and 10 times (Table 4 assays 2 and 1) the corresponding controls; presumably this is due to the protective effect of the NaCl. Indeed, on addition of NaCl to chromatin irradiated in water the activation was higher (Table 4, assay 7) *viz.* 8 times the control value (assay 5). In this case, the activity of the soluble fraction was about 30% of that of the whole suspension. This relatively high value suggests that once a region of the chromatin has suffered radiation damage the DNA-protein linkage in that region becomes more labile in 0.05 M NaCl (*cf.* Hagen [21], Robinson *et al.* [22] and Lloyd and Peacocke [23]) and hence the damaged chromatin becomes both more soluble and more active as a template.

From these results we conclude that the increased incorporation in chromatin after irradiation is due probably to the production of new initiation sites in the regions to which added polylysine would bind, *i.e.* in the zones of "accessible" DNA phosphates.

Whether or not new sites are produced elsewhere in the DNA component, but are inaccessible to the DNA polymerase, is uncertain. DNA was prepared from samples of control and irradiated chromatin and values of template activity were compared with that of calf thymus DNA treated in a similar way. Table 3 shows that all three samples had similar activity. This means that either new enzyme-binding sites are indeed produced only in the accessible zones or, if they are produced elsewhere, they are in the main active enzyme-binding sites rather than inactive (as in the accessible zones). Under the conditions used for the assay of DNA, an increase in the number of active sites cannot be estimated directly (as there is an excess of enzmye binding sites over enzyme molecules). Experiments are in progress to find whether new binding sites are produced in the inaccessible zones of DNA in chromatin.

No previous studies have been made on the effect of ionising radiation on the template activity of chromatin for DNA polymerase I although the effect on transcription has been examined [24]. Most of the previous work has been concerned with changes in molecular weight (*e.g.* Lloyd and Peacocke [25]) or in intramolecular linkages (*e.g.* Errera [26], Cole and Ellis [27] and Itzhaki [20]). However, it is generally agreed that in chromatin, the protein partially protects the DNA from the indirect effect of irradiation. The present result with added polylysine shows that a more complete masking of the chromatin DNA by the polylysine totally inhibits radiation-induced activation for DNA polymerase. Further, the large proportion of radiation-produced active enzyme-binding sites in chromatin when compared to DNA ($\approx 98\%$ compared to $\approx 30\%$) argues against the presence of extensive wholly free DNA zones in chromatin as these would presumably be damaged preferentially in a way similar to that of pure DNA. Instead it supports the previous conclusion (Itzhaki [18]) that in chromatin, most or all of the DNA is associated with protein.

CONCLUSIONS

The present results agree with those of previous workers in showing that chromatin has a low template activity with DNA polymerase I when compared to DNA. Billen and Hnilica [4] and Gurley *et al.* [3], who studied reconstituted nucleohistone, proposed that the low activity was due to the insolubility of the complex in the assay system, the former showing that the decrease in template activity paralleled the decrease in solubility of the complex. However, Wood *et al.* [5] showed that inhibition occurred even under assay conditions in which their chromatin remained soluble. Further, Schwimmer and Bonner [2] and Brown *et al.* [7—9] obtained low template activity using preparations of "soluble" chromatin. The former authors reported also that the DNA produced had the same sedimentation coefficient as that produced on a DNA template. Brown *et al.* [7—9] found that specific polyanions released the DNA template restriction in chromatin and attributed this to interaction with nuclear components such as histones. However, Slater and Loeb [6] showed that *E. coli* exonuclease III activated chromatin and suggested that the chromosomal proteins, including histones, may not control initiation and replication of DNA.

After this study was completed, Umiel and Plaut [28] published experiments on the priming capacity of fixed nuclei and chromosomes for DNA polymerase I. Using radioautographic techniques they showed that the activity increased after treatment with 0.1 N HCl, which presumably released histones, and decreased after addition of polylysine. In agreement with our conclusions, they suggest that inaccessibility of the DNA in chromatin may possibly account for its low activity.

As far as we know, the present study is the first to assay separately both the soluble and insoluble fractions of chromatin under various conditions as well as the first to attempt to relate initiation sites *in vitro* to specific zones of the chromatin. Of our four possible explanations for the lower template activity of chromatin compared to DNA, we have excluded two, a smaller number of initiation sites in the chromatin DNA or a lower rate of DNA synthesis per enzyme molecule. As for the other two possibilities, our results show that although the insolubility of the chromatin in the assay system does play a very limited role, the major factor responsible for the low template activity of chromatin is the inaccessibility of the initiation sites to the DNA polymerase I. Because of the several different procedures used for preparing chromatin, it is difficult to compare directly our results with those of other workers. However, our general conclusion, that inaccessibility is the major factor (despite the insolubility of the bulk of our chromatin), agrees with that of those workers who used only "soluble" chromatin.

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