Accessibility of DNA in Chromatin to DNA Polymerase and RNA Polymerase
(chromatin/DNA polymerase/RNA polymerase)

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ABSTRACT The accessibility of DNA in chromatin to both exogenous DNA polymerase and RNA polymerase is slight when compared to isolated DNA. DNA in extracted chromatin is somewhat more accessible to these enzymes than is DNA in the chromatin of isolated nuclei; and the DNA template of chromatin is more accessible to DNA polymerase than to RNA polymerase. In these experiments we have given much attention to the technique of scintillation counting, since artifacts arising in this procedure can lead to erroneous conclusions.

Since DNA in chromatin is complexed with proteins, accessibility to DNA by enzymes and other substances is restricted. The role of histones in restricting access to DNA in chromatin was first observed by staining with basic dyes. Staining of the cell nucleus led Flemming (1) in 1882 to coin the term chromatin to designate the material that takes basic stains, and Flemming knew that chromatin contains DNA. The combination of DNA with crystal violet was shown by Feulgen (2) in 1913 to be stoichiometric: one molecule of dye for each phosphoric acid group of DNA. Later (1951), in a quantitative study of the reaction of isolated chromatin with crystal violet, Mirsky and Ris (3) found that only 8% of the phosphoric acid groups of DNA combined with dye but that removal of histone from the chromatin opened access to DNA so that 30% of the phosphoric acid groups of DNA combined with dye. Blocking of DNA by histones was later (1962) shown by Huang and Bonner (4) in experiments with RNA polymerase. Recently we have found that only a small part of the DNA in chromatin is freely accessible to DNase (5).

We report in this paper determinations of the accessibility of DNA in chromatin to exogenous DNA polymerase and DNA-dependent RNA polymerase. In our experiments we have studied chromatin in isolated nuclei and also in extracted chromatin (deoxyribonucleoprotein, DNP). Most of the previous work in this field has been on DNP, in which it is possible to replace certain components of chromatin, but the purpose of all these investigations is, of course, to understand chromatin as it is in the nucleus. In the experiments we are now reporting we have compared the activities of both exogenous DNA and RNA polymerases on free DNA and on DNA in both thymus nuclei and extracted chromatin. The comparison shows clearly that only a very small fraction of the DNA in a nucleus is accessible to either DNA or RNA polymerase; DNA in DNP is slightly more accessible. We have used exogenous polymerase in these experiments so that availability of the DNA template should not be obscured by enzymatic variability. It will be seen that we have given much attention to the technique of scintillation counting, for a mistake in this procedure can lead to erroneous conclusions.

MATERIALS AND METHODS

Materials

The preparation of nuclei isolated from calf thymus (6) and liver (7) in 0.25 M sucrose–3 mM CaCl₂ has been described. DNP (extracted chromatin) was prepared from thymus nuclei by the method of Paul and Gilmour (5, 8). Highly polymerized native calf-thymus DNA was obtained from Worthington Biochemicals Corp. Nucleotide triphosphates were products of Schwarz/Mann, who also supplied [³H]TTP (specific activity, 18 Ci/mmol) and [³H]UTP (specific activity, 15 Ci/mmol). Highly purified Escherichia coli DNA polymerase was prepared and assayed according to Richardson et al (9). It was supplied by General Biochemicals at a specific activity of 5000 units/mg of protein. RNA polymerase from E. coli B was prepared by the method of Chamberlin and Berg (10). It was purchased from General Biochemicals at 2000 units/mg of protein. Hyamine (T. M. Rohm and Haas) was obtained from Nuclear Associates, Inc.

Experiments with DNA polymerase

Incubation. Enzymatic activity was assayed with the following reaction mixture adapted from Englund (11); 50 mM Tris-HCl buffer (pH 7.4), 5 mM MgCl₂, 1 mM 2-mercaptoethanol, dATP, dCTP, dGTP, and TTP each at 0.04 mM, 10 μCi/ml of [³H]TTP, usually 20 μg of DNA per ml, and 4 units of DNA polymerase per ml. Incubations were done at 37° for 30 min with occasional stirring in a minimum volume of 0.75 ml. The reaction was terminated by chilling to 0° and precipitating the DNA and proteins in 5% trichloracetic acid–1% sodium pyrophosphate. Incorporation of radioactivity into an acid-insoluble product was then measured by liquid scintillation counting of triplicate 0.2-ml aliquots from each incubation. Counts per min from control incubations containing no DNA template were subtracted from each experimental value. In each case these counts were about 0.5% of those found with isolated DNA as was found if DNA polymerase was omitted from the incubations.

Scintillation Counting. Initially, we used a generally accepted assay procedure that has been described by numerous investigators (11). These workers did not vary the concentration of DNA template in the reaction mixture. Rather, they used a fixed quantity of template to evaluate procedures concerned with the fractionation and purification of DNA.
polymerase or to study the effects of variables other than template concentration on enzymatic activity. The assay procedure used most frequently in these studies and initially adopted by us is to bring the incubation mixture to a final concentration of 5% trichloroacetic acid-1% sodium pyrophosphate to precipitate DNA and protein and then to pass the suspension through a glass-fiber filter. The filter is washed to remove acid-soluble radioactivity, dried, and counted to determine acid-insoluble radioactivity. We used Whatman GF/C glass-fiber filters saturated with 5% trichloroacetic acid-1% sodium pyrophosphate. After filtration, they were washed with 15-ml aliquots of the trichloroacetic acid-sodium pyrophosphate solution, 95% alcohol, and ether. The filters were air dried, placed in 15 ml of a toluene-based scintillation fluid, and counted. In this procedure more than 98% of the radioactivity remained associated with the filter. When we used this assay system to determine the effect of varying the concentration of DNA template in the incubation mixture, we obtained the result illustrated by curve B in Fig. 1. Enzymatic activity reached a maximum at about 20 μg/ml of DNA template and decreased fairly sharply at higher concentrations of DNA. This result is essentially the same as that obtained by Schwimmer and Bonner (12) using a similar assay procedure. Is this maximum characteristic of the DNA polymerase reaction or is it an artifact due perhaps to self-absorption by DNA in the scintillation counting technique? To answer this question, we added increasing amounts of nonradioactive DNA to a constant known amount of tritiated DNA synthesized with 20 μg/ml of template DNA. The counts recovered were inversely proportional to the added DNA and mimicked curve B in Fig. 1. Therefore, the apparent decrease in enzymatic activity seen at DNA concentrations greater than 20 μg/ml is an artifact of the scintillation counting procedure. Most likely the explanation is related to self-absorption occurring as increasing amounts of DNA are collected on the glass filters.

In order to eliminate scintillation counting artifacts that can occur using the "standard" procedure that we originally adopted, we used hyamine to dissolve the nucleic acid collected on the glass-fiber filters. After the filters were washed and dried as previously described, each filter was placed in a scintillation vial containing 0.5 ml of hyamine and gently agitated in a 70° water bath for 30 min or kept overnight at 38°. 15 ml of scintillation fluid was then added, and the filters were counted as before. In contrast to the previous finding, essentially all of the radioactivity was found in the scintillation fluid. When DNA in concentrations up to 400 μg/ml was precipitated in 5% trichloroacetic acid collected on filters, washed, dried, and dissolved in hyamine, it did not quench the counts in an added known amount of tritiated toluene. Thus all samples assayed in this manner are counted at the same efficiency, about 20%, irrespective of the amount of DNA used as template.

When we repeated DNA polymerase experiments, varying the concentration of DNA template, and used hyamine to dissolve the DNA, the results were entirely different from those obtained when the radioactivity remained immobilized on the glass filters. A plateau of enzymatic activity was reached at about 100 μg/ml (Fig. 1, curve A). This is comparable to results reported by some other investigators (13-15).

Another assay procedure was used in experiments with isolated DNA and found to corroborate the results obtained with the hyamine counting technique. Bollum has described (16) a procedure in which aliquots of incubation mixtures are spotted directly onto dry filters, which are then dropped into a single beaker of trichloroacetic acid. Thus the nucleic acid is precipitated within the filter rather than being precipitated in a test tube and then collected on the filter. The filters are washed, dried, and counted as in other procedures. When we used this technique to repeat experiments varying the concentrations of template DNA, we found we could reproduce and corroborate the results obtained with the hyamine procedure (curve A, Fig. 1). Apparently counting efficiency is constant over a wide range of DNA concentrations when the DNA is precipitated within the glass-fiber filters. Unfortunately, when nuclei are used as the source of template DNA they tend to wash off or out of the filters. Thus Bollum's technique was not suitable for most of our experiments.

If, instead of isolated DNA, nuclei or DNP serve as the template for DNA polymerase, hyamine is still required to dissolve the template before scintillation counting. Omission of hyamine leads to results that are lower than those obtained when the nuclei are dissolved. However, even at very high concentrations, nuclei do not act as quenching agents when dissolved in hyamine and mixed with a known amount of tritiated toluene. Thus quenching by either protein or DNA is not a factor in this counting procedure.

Template Activity of DNA in Chromatin. Curves C and D in Fig. 1 illustrate the effectiveness of intact nuclei and DNP when used as the source of template DNA for DNA polymerase. Neither template demonstrates a maximum or even a well-defined plateau value of enzymatic activity as its con-
centration is increased. Both are rising slowly even at 200 
µg/ml of DNA, and both are only slightly accessible to DNA 
apolymerase when compared to isolated DNA. At 100 
µg/ml of DNA, the beginning of the plateau found with isolated 
DNA, the template activity of DNP is about 7%, while that 
of nuclei is 4%, of the value found with DNA. The corre-
responding values at 200 µg/ml of DNA are 14% for DNP and 
9% for nuclei. Additional experiments showed that nuclei 
isolated in 0.01 M citric acid exhibited essentially the same 
template activity as those prepared in isotonic sucrose.

An interesting and significant addition to these data was 
made when liver nuclei replaced thymus nuclei as the source 
of DNA in the assay system. The template activities of these 
nuclei were indistinguishable from each other in spite of the 
relatively high content of nonhistone protein in liver chromo-
matin (3, 17).

It should also be noted that when DNP was used as the 
source of template DNA, it was entirely insoluble during the 
in incubation with DNA polymerase. At concentrations above 
80 µg of DNA per ml some of the DNP was obviously in-
soluble in the incubation medium. To find out how much DNA, 
if any, was in solution, we centrifuged incubation mixtures 
containing different samples of DNP, hydrolyzed any DNA 
in the supernatant with 0.5 M perchloric acid, and measured 
the absorbance at 265 nm. As determined by this procedure, 
less than 1% of the DNA in DNP was in solution during the 
in incubation with the enzyme. Almost certainly this is also 
true in experiments conducted by others, since only a small 
amount of divalent cation (18), here 5 mM, is necessary to 
precipitate DNP. Furthermore if DNP is precipitated in 
0.14 M NaCl it appears more “clumped” than when pre-
cipitated by the divalent cation in the incubation mixture, 
yet its template activity is only slightly reduced. Of course 
the chromatin within isolated nuclei is insoluble and remains 
so during our experiments. If the nuclei are broken mechani-
cally, the template activity of the preparation is about 80% 
of the value found with isolated nuclei. This is strong evidence 
that the nuclear membrane is not limiting the availability of 
DNA polymerase to the chromatin of isolated nuclei. Thus 
the observation that the DNA in chromatin is only slightly 
accessible to DNA polymerase, when compared to isolated 
nuclei, is not due to the enzyme failing to penetrate into the 
nuclei.

The results that have just been presented were obtained 
with the standard assay system described under Incubation. 
Varying the concentration of nucleotide triphosphates, the 
in incubation time, and the concentration of enzyme did not 
alter the main conclusion drawn from Fig. 1: the DNA of 
either nuclei or DNP, as compared to isolated DNA, is only 
slightly accessible to DNA polymerase. Incubation times of 
15–120 min did not change the template activity of chromatin 
relative to isolated DNA. This was true when thymus nuclei 
or DNP or liver nuclei were used as the source of template 
DNA. Concentrations of DNA polymerase up to 5-times 
greater than that used in Fig. 1 also did not affect the relative 
template activity of DNA in chromatin. Therefore, the ac-
cessibility to DNA polymerase observed in these experiments 
is a reflection of the structure of the chromatin template, not 
of parameters of the assay system used to measure it.

Experiments with RNA polymerase

Incubation. Enzymatic activity was assayed with the follow-
ing incubation mixture, which is modeled after that of Cham-
berlin and Berg (10): 40 mM Tris-HCl buffer (pH 7.9), 
0.15 M KCl, 1 mM MnCl₂, 4 mM MgCl₂, 10 mM 2-mercap-
toethanol, ATP, CTP, GTP, and UTP each at 0.12 mM, 10 
µCi/ml of [³H]UTP, usually 20 µg of DNA per ml, and 4 
units of RNA polymerase per ml. Incubations were done at 
37° for 20 min with occasional stirring in a minimum volume of 
0.75 ml. The reaction was terminated and assayed as 
described for DNA polymerase.

Scintillation Counting. The problems of assaying the in-
corporation of a radioactive nucleotide into an acid-insoluble 
product are essentially the same whether RNA or DNA 
polymerase is used. Thus the procedure most commonly 
described by other investigators (11) for assaying RNA 
polymerase activity is identical to that which has been most 
often used for the DNA polymerase system: the nucleic acid 
is precipitated in 5% trichloroacetic acid, collected on a 
filter, washed to remove acid-soluble radioactivity, dried, and 
counted. As was the case with DNA polymerase, most other 
investigators did not vary the concentration of template in 
the reaction mixture. Therefore we repeated the experiments 
first done using DNA polymerase with RNA polymerase and 
obtained the same general results (Fig. 2) as were presented 
in Fig. 1. When the nucleic acid was collected on glass-fiber 
filters, a maximum was obtained at about 25 µg/ml of DNA 
curve B, Fig. 2) and template activity decreased at higher 
concentrations of DNA. However if the nucleic acid was dis-
solved from the filter with hyamine, the plateau of template 
activity shown in curve A of Fig. 2 was observed. Thus 
the same experiments and arguments are relevant for both DNA 
and RNA polymerase assays: the procedure where nucleic 
acid remains immobilized on the filters is subject to an arti-
fact of scintillation counting as the concentration of DNA is 
increased. However, the use of hyamine to dissolve the nu-
cleic acid eliminates this artifact. There have been several 
investigators (20–22) who have varied the concentration of 
DNA template in the RNA polymerase reaction, dissolved 
the nucleic acids and proteins, and then proceeded with the 
scintillation counting. Their results are essentially the same 
as reported in Fig. 2, curve A. Furthermore Bollum’s tech-
nique (16), to which we have already referred, also corrobo-
rates the results of Fig. 2, curve A. Unfortunately, this tech-
nique cannot be used if nuclei replace isolated DNA in the 
in incubation. And as was the case with DNA polymerase, 
when nuclei or DNP are used as templates for RNA polymerase, 
hyamine must be used to dissolve the template before scin-
tillation counting.

Template Activity of DNA in Chromatin. Curves C and D in 
Fig. 2 illustrate the effectiveness of nuclei and DNP when 
used as the source of template DNA for RNA polymerase. 
Both templates are only very slightly accessible to the en-
zyme. Increases in template activity are not observed when 
the concentration of DNA is increased. DNA in nuclei ex-
hibits about 2% of the template activity of isolated DNA, 
whereas the enzyme for DNP is about 4%. Liver nuclei (not 
illustrated) assay at about 1% relative to isolated DNA. Thus 
thymus and liver nuclei have essentially the same accessibility 
toward RNA polymerase, as was the case in experiments with 
DNA polymerase. Mechanically broken thymus nuclei have 
the same template activity as unbroken nuclei. Thus, as was 
true with DNA polymerase, the limited accessibility of DNA 
in these nuclei is not due to the enzyme failing to penetrate 
into the nucleus. It should also be noted that when DNA
in DNP is used as a template, it is completely insoluble during the incubation with RNA polymerase.

In order to eliminate the possibility that RNase activity was degrading the RNA synthesized in these experiments, the virtual absence of such activity was demonstrated in the following manner: Tritiated RNA was synthesized using nuclei depleted of lysine-rich histone. (This increased the template activity of the nuclei, as will be reported in a future paper.) The nuclei were centrifuged and the supernatant was assayed for [3H]RNA before and after an incubation with added intact thymus or liver sucrose nuclei (40 μg of DNA per ml). After 20 min at 37°C, 99% of the counts in the supernatant representing [3H]RNA were still acid-insoluble, and thus the RNA had not been hydrolyzed by RNase activity.

As was found for DNA polymerase, when enzyme concentration, incubation time, and the concentration of nucleotide triphosphates were varied, they did not affect the conclusions drawn from Fig. 2. The accessibility of DNA in nuclei to RNA polymerase, relative to isolated DNA, did not change when more than three times the enzyme concentration was used. It should be noted that even when higher template activities were obtained, as when histones were extracted from nuclei, the template activity relative to isolated DNA was independent of the concentration of enzyme. Obviously the possibility of a significant factor at the extremely low template activity of intact nuclei. However when we used histone-depleted nuclei whose template activity was considerably higher, the relative template activity of these nuclei did not change with incubations ranging from 15–60 min.

**DISCUSSION**

We have just described observations on the effects of exogenous polymerases on the template activity of DNA in chromatin. How are these observations to be related to the template activities of DNA with respect to replication and transcription in nuclei when these activities depend upon the endogenous polymerases? First, *transcription*: autoradiographs of RNA synthesis in calf-thymus nuclei (23) and *Chironomus* polytene chromosomes (24) show that only a very small part of the DNA is transcribed—in the former mainly in the relatively small amount of diffuse chromatin, in the latter mainly in the puffs. It is not generally supposed that the limited transcription in such cases is due to lack of RNA polymerase, but this possibility has not been excluded. This is a possibility that should be considered, for RNA polymerase can have an exceedingly short turnover time (25). In experiments on the effect of exogenous RNA polymerase on transcription in extracted chromatin, numerous reports (26) have shown that the meager RNA synthesis is due to inaccessibility of the DNA template. The experiments using exogenous polymerase described in the present report show that also for chromatin in nuclei only a very small part of the DNA template is accessible. In these experiments the results were the same for thymus and liver nuclei. Since the ratio of nonhistone (or acidic) protein to histone is much greater in liver than in thymus nuclei (3, 17), it does not appear that the over-all nonhistone protein is a significant factor in template restriction. Application of exogenous RNA polymerase to isolated polytene chromosomes shows, according to a recent report (27), that in this material too, inaccessibility of the template restricts transcription. The observations on the marked restriction of template activity in transcription are, of course, in accord with the results of DNA–RNA hybridization experiments that have shown that only a small part of the DNA in a nucleus is transcribed.

**DNA replication**

Although but a small fraction of the DNA in a nucleus is transcribed, all of the DNA of a nucleus is replicated in each cell cycle. According to a report by Schimmer and Bonner (12), this difference between replication and transcription can be accounted for by what appears to be the far greater accessibility of DNA in chromatin to DNA polymerase than to RNA polymerase. Schimmer and Bonner (12) reported that “although nucleohistone is totally ineffective in the support of DNA-dependent RNA synthesis .... nucleohistone is nearly as effective in support of DNA synthesis as is deproteinized DNA” at a high concentration of the DNA template. In a recent review (28) the situation was put this way: “Native chromatin, even if inactive in support of RNA synthesis by RNA polymerase, is active in support of DNA synthesis by DNA polymerase.” In another review (29) it was concluded that “Any model for the effect of histones on DNA replication must account for the findings of Schimmer and Bonner that native chromatin (with histones intact) can be used for DNA synthesis by DNA polymerase.”

In a recent paper (30), we referred to the experiments of Schimmer and Bonner because we had found that DNase in sufficient concentration can in the course of time digest practically all the DNA in an isolated nucleus (5). It seemed to us noteworthy that although DNA in chromatin is only slightly accessible to RNA polymerase, our observations and those of Schimmer and Bonner (12) indicated that the DNA of chromatin is far more accessible to DNase and DNA.
polymerase. When we repeated their experiments, which presented no difficulty, we were at first satisfied with their conclusion. This can be understood by inspection of curves B and C, Fig. 1; as the template concentration increases, the template activity of chromatin approaches that of isolated DNA as represented by curve B. The approach is due to the fact that curve B falls rapidly with increasing template concentration. This fall in curve B (compared with curve A) depends, as we have shown in this paper, on an artifact of scintillation counting. It can now be seen that because of this artifact Schwimmer and Bonner reached an erroneous conclusion.

When the curves (C and D, Fig. 1) for DNA replication in chromatin are compared with the true curve (A) for replication of isolated DNA, it is clear that the DNA template in chromatin is only slightly accessible to DNA polymerase. The template is, however, significantly more accessible to DNA polymerase than it is to RNA polymerase (Fig. 2). For both polymerases the accessibility of DNA in thymus nuclei is about the same as it is in liver nuclei, despite the relatively large amount of nonhistone protein in liver chromatin (3, 17).

It should be noted that the accessibility of DNA in chromatin to DNase is also the same in liver nuclei as it is in thymus nuclei (30). The marked difference between the polymerases and DNase in their action on chromatin is that the latter at a high concentration of enzyme and in the course of time finally digests practically all of the DNA (5). This result is probably made possible by a change in structure of chromatin due to the decomposition of DNA.

If, as we have found, DNA in the chromatin of interphase nuclei is only slightly accessible to exogenous DNA polymerase, it seems likely that there is a change in chromatin at the time of replication. Two recent reports indicate that there is a change in chromatin at the time of replication: Pederson (31) found that such chromatin was more accessible to digestion by DNase; and Tan and Lerner (32) found that single-stranded DNA was not detected immunologically in the nuclei of multiplying lymphocytes during phase G, but was "abundantly present" in phase S. It may well be that this single-stranded DNA is accessible to a DNA polymerase present in the phase S nucleus.

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