Control of RNA synthesis by chromatin proteins

Howard Cedar, Adinah Solage and Fukiko Zurucki

Department of Molecular Biology, The Hebrew University-Hadassah Medical School,
Jerusalem, Israel 91000

Received 2 April 1976

ABSTRACT

The effect of chromatin proteins on template activity has been studied. Using both *E. coli* RNA polymerase and calf thymus polymerase B we have measured the number of initiation sites on chromatin and various histone-DNA complexes. Chromatin can be reconstituted with histone proteins alone and this complex is still a restricted template for RNA synthesis. The removal of histone fl causes a large increase in the template activity. Chromatin is then treated with Micrococcal nuclease and the DNA fragments protected from nuclease attack ("covered DNA") are isolated. Alternatively, the chromatin is titrated with poly-D-lysine, and by successive treatment with Pronase and nuclease, the DNA regions accessible to poly-lysine are isolated ("open DNA"). Both fractions were tested for template activity. It was found that RNA polymerase initiation sites are distributed equally in open and covered region DNA.

INTRODUCTION

It is generally believed that the proteins of chromatin play a major role *in vivo* in limiting the template activity of the DNA to which they are bound, and that this limitation is related to cellular differentiation. Chromatin isolated from eukaryotic nuclei retains the information required to direct the transcription of RNA specific to the tissue from which the chromatin was extracted (1, 2, 3). We can only speculate at this time as to the nature of the controlling elements, but studies on the reconstitution of chromatin proteins with DNA suggest that the non-histone proteins include specific transcriptional control factors (4, 5, 6). Furthermore it has previously been demonstrated that there is a relationship between the distribution of proteins on DNA and the biological role of chromatin in the transcription process. In the case of the globin gene of avian blood cells, the actively transcribed genes are partially covered by protein in a site-specific manner (7).

Chromatin is a restricted template for RNA synthesis in vitro and it has been demonstrated that this is mainly due to a decrease in the number of available RNA polymerase initiation sites on this template (8). The mechanism for restriction is not well understood. Whereas Bonner et al. (9) have suggested that RNA synthesis is controlled by both histone and non-histone protein, Koslov and Georgiev (10) have suggested that histone proteins, mainly histone fl are responsible for this repression. In this paper we investigate the mechanism of this transcriptional control using an assay which specifically measures initiation sites. We find that histone alone, when added to the DNA under equilibrium conditions can account for the restriction of RNA synthesis on chromatin. Furthermore the RNA initiation sites which are repressed in chromatin are not localized to those regions of chromatin which are covered by protein.

MATERIAL AND METHODS

(a) Material - Nucleoside triphosphates were purchased from Cal-Biochem and P L Biochemicals. (^3H) UTP (40 Ci/mmol) was obtained from New England Nuclear Corp. Poly D-lysine hydrobromide (PDL) was obtained from Sigma. It had an average molecular weight of 70,000. Histones fl, f2a1, f2a2, f2b and f3 were a gift from Dr. Michael Bustin. They were shown to be greater than 95% pure by acrylamide gel electrophoresis. Purified Micrococcal nuclease was purchased from Worthington Biochemical Co. and Pronase B was from Cal-Biochem. Total histone was prepared from calf thymus by acid extraction (11).

E. coli RNA polymerase was prepared from frozen late-log phase cells by the procedure of Berg et al. (12) yielding a fraction V enzyme with a specific activity of 700 units/mg. One unit of activity, as defined by these authors is equivalent to the incorporation of 1 nmol of ATP in 10 min. In order to calculate the number of RNA polymerase molecules, we assumed a maximum specific activity of 1200 units/mg and a molecular weight of 4.7×10^5 (12).

Calf thymus RNA polymerase B was purified from frozen calf thymus according to the procedure of Keding et al. (13) and Keding and Chambon (14). In this procedure the enzyme is freed from the vast amount of DNA by sonication followed by protamine sulfate precipitation. Separation of calf thymus polymerase A and B was accomplished by DEAE-cellulose chromatography, which results in a three-

fold purification from the original lysate. Further purification was done using phosphocellulose chromatography which gave an enzyme specific activity 6 times greater than that of the DEAE fraction. This latter enzyme was used in all of the studies presented in this paper. One unit of activity is equivalent to the incorporation of 1 nmole UTP in 10 min using the assay conditions described for this enzyme (13). The enzyme was completely sensitive to α amantin.

(b) Preparation of DNA and chromatin - Calf thymus and chicken erythrocyte chromatin were prepared from Triton-washed nuclei by a stepwise reduction in ionic strength as previously described (1). The final preparation was sheared to an average DNA molecular weight of $6-8 \times 10^6$ in a Waring Blendor (1 min at 90V); but was not sonicated. Chromatin prepared in this way had a protein/DNA ratio of 1.3 g/g and was stable for up to 6 weeks at 4 C. DNA was prepared from isolated nuclei as described (1).

(c) Assay for RNA polymerase initiation sites - RNA polymerase (either bacterial or eukaryotic) and template were incubated at 37 C in 0.5 ml containing 10 mM Tris-HCl (pH 7.9), 1 mM $MnCl_2$, 0.08 mM each of ATP and GTP and 0.02 mM (^3H)UTP (500 cpm/pmole). This initiation reaction (15 min) was stopped by the addition of 0.16 ml of 1.6 M $(NH_4)_2SO_4$. This step is important to prevent further chain initiation by enzyme molecules (15). Propagation in high salt was then started by the addition of CTP (final concentration 0.063 mM). Both DNA and chromatin are visibly soluble at every stage in this procedure. Incorporation into RNA was determined by TCA precipitation. In order to determine the absolute number of initiated RNA polymerase molecules, the average molecular weight of the newly synthesized RNA is measured by sucrose gradient ultracentrifugation (8). The relative number of initiation sites can be determined from total nucleoside triphosphate incorporation alone, since the size of the RNA synthesized under these conditions is the same for all templates used in this study. In the case of *E. coli* RNA polymerase, the number average size of the RNA was 800 nucleotides. For calf thymus RNA polymerase B it was 600 nucleotides.

(d) Preparation of "open" and "covered" DNA from chromatin - About half of the DNA in chromatin resists digestion by the enzyme Micrococcal nuclease.

This fraction of DNA (covered DNA) is prepared in large quantities as follows: 100 ml of a solution of 0.1 mM CaCl_2 - 2 mM Tris-HCl (pH 7.9) are added dropwise with stirring to 100 ml of chromatin at a concentration of 0.5 mg/ml (DNA content). Micrococcal nuclease is then added to a concentration of 8 $\mu\text{g}/\text{ml}$ and the reaction is incubated at 37 C for 1 hr with gentle stirring. Toward the end of the reaction the protected DNA precipitates and is collected by centrifugation at 10,000 rpm in a Sorvall SS-34 rotor. This precipitate contains half of the original complement of DNA and virtually all of the chromatin proteins. The precipitate is suspended in 0.01 M Tris-HCl (pH 7.9), 0.4 M NaCl, 0.005 M EDTA and 0.5% sodium dodecyl sulfate and freed of protein by extraction with phenol-chloroform. The covered DNA is then precipitated from the aqueous phase by the addition of two volumes of ethanol.

The regions of DNA in chromatin susceptible to nuclease attack and titratable by poly-D-Lysine (open DNA) are prepared as follows: 100 ml of a solution containing 70 μmole of poly-D-Lysine in 2 mM Tris-HCl (pH 7.9) are added dropwise with stirring to 100 ml of chromatin, DNA content 0.5 mg/ml (150 μmole of DNA) to yield a (lysine) / (DNA phosphate) ratio of 0.46. Pronase is then added to a concentration of 8 U/ml and the reaction is incubated at 37 C for 3 hr and then at 4 C for 16 hr to permit autodigestion of the Pronase. This reaction results in complete digestion of the chromatin proteins leaving in solution a complex of poly-D-lysine bound to DNA regions previously accessible in intact chromatin. The DNA complexed to poly-D-lysine is now isolated by nuclease digestion in a manner identical with that described for the preparation of native covered DNA, except that the limit digest was first adsorbed on hydroxyapatite in 0.5 M NaCl and the DNA eluted with 0.5 M phosphate buffer (pH 6.8).

RESULTS

Initiation sites on reconstituted chromatin - We have previously shown that chromatin is a restricted template for both *E. coli* RNA polymerase (8) and calf thymus polymerase B (16). This restriction of initiation sites is most probably caused by chromatin proteins, and the restriction is preserved even after the proteins are disassociated from the DNA and then reconstituted by stepwise salt-urea dialysis. Both duck reticulocyte chromatin (17) and calf thymus chromatin (Table 1)

TABLE 1. Template activity of reconstituted DNA-protein complexes

Template	Template activity	
	<i>E. coli</i> RNA polymerase	Calf thymus RNA polymerase
DNA	100	100
Chromatin	7	6
DNA-histones	8	7
DNA-(f2b, f2a1, f2a2, f3)	21	-
DNA-f2b	25	20
DNA-f1	30	-

Chromatin or DNA with histones (1.3 g histone/g DNA) were put in 5 M urea, 2M NaCl, and then dialyzed successively for 80 min each in 5 M urea containing 1.2 M NaCl, 1.0 M NaCl, 0.8 M NaCl and 0.6 M NaCl. It was then dialyzed against 0.6 M NaCl for 80 min and finally against 0.1 mM EDTA overnight. All dialysis buffers contained 1 mM Tris-HCl (pH 7.9) and 10 mM β -mercaptoethanol was included in all buffers except the last. The number of initiation sites (template activity) was measured as described in Material and Methods and the results are expressed as a percentage of activity obtained using DNA as template. Each assay was done with either 2 units of *E. coli* RNA polymerase or 1.5 units of calf thymus RNA polymerase B. Each result is an average of activity obtained with different amounts of template. RNA polymerase was always in excess. For the *E. coli* enzyme we used 3 units/mg template (Cedar and Felsenfeld, 1973). For the calf thymus enzyme the ratio was 0.3 units/ μ g template (Cedar, 1975). All histone complexes were 45 - 55% digestable with Micrococcal nuclease.

could be reconstituted in this way and still retained the same number of initiation sites relative to DNA

With the knowledge that template activity is conserved when chromatin proteins are added back to DNA, one can ask which proteins are responsible for this restriction. In this light we prepared histone proteins from calf thymus chromatin by acid extraction. This histone preparation contained over 96% histone proteins. When these proteins were added back to DNA (1.3 g histone/g DNA) by the salt-urea reconstitution procedure we obtained the same number of initiation sites as native

chromatin. Similar results were obtained using chicken erythrocyte chromatin. Both native erythrocyte chromatin and a DNA histone complex prepared by reconstitution had a relative template activity of 2%.

Thus histone proteins alone seem to account for all of the template restriction of chromatin. This restriction required the presence of all of the histones. DNA reconstituted with individual histone f1 or f2b had relative template activity of 30%, which is about five times higher than the activity of chromatin (Table 1). Other complexes constructed from other individual histones also had a relative template activity of about 30% (data not shown). As shown in Table 1 a complex containing all of the histones except f1 had a relative template activity 3-4 times higher than native chromatin. Preliminary results with other groups of four histones indicate that only complexes containing all five histones can restore the template activity of chromatin. It should be noted (Table 1) that similar results were obtained with both *E. coli* RNA polymerase and calf thymus polymerase B.

Initiation sites on open and covered regions - Since chromatin proteins protect only 50% of the DNA and since these proteins restrict the initiation of RNA synthesis, it is of interest to know whether there is a relationship between DNA covering and inhibition of template activity. To this end we prepared open region DNA and covered region DNA from chromatin; covered DNA was obtained by purifying DNA from protein after digestion of the chromatin with Micrococcal nuclease. Open DNA was made by titrating chromatin with PDL followed by digestion of all the native proteins using Pronase B. The resulting PDL-DNA complex was digested with Micrococcal nuclease to yield open region DNA. We then tested both types of DNA for the number of initiation sites. The results are shown in Fig. 1.

Since both open and covered DNA have an average molecular size of 100-200 nucleotide pairs (18) the usual technique for measuring template activity cannot be used. Because of the small size only short RNA chains are synthesized and this RNA is too short to be analyzed by sucrose gradient centrifugation. An alternative method for measuring RNA polymerase initiation sites is to titrate a fixed amount of RNA polymerase with varying amounts of template (8). As DNA is added the amount of RNA initiations increases until a plateau is reached. At this titration point, the number of initiation sites is equal to the number of RNA polymerase molecules.

A typical experiment using duck reticulocyte chromatin is shown in Fig. 1. Using a fixed amount of *E. coli* RNA polymerase open region DNA and covered region DNA as well as total sonicated duck DNA reach a plateau at the same level of

Figure 1. Titration of *E. coli* RNA polymerase with open and covered region DNA. Increasing amounts of sonicated duck DNA (●), open region DNA (▲) or closed region DNA (■) were added to 0.6 units of RNA polymerase. Incubation conditions were the same as those described in Materials and Methods. After 20 min of propagation in high salt the mixtures were assayed for incorporation into tri-chloroacetic acid precipitable material. The data are expressed as pmol UTP incorporated in 20 min. Incorporation in absence of template was less than 1 pmol.

added template. Similar results were obtained using open and covered regions prepared from calf thymus chromatin.

The amplitude of the plateau is different for each template, reflecting the difference in size of the three templates. Whereas the average molecular size of sonicated DNA is 500 base pairs, the weight average degree of polymerization of open and covered DNA were 210 and 150 respectively (7). Since the RNA synthesized from these templates varies with the size of the DNA, we obtain plateaus at different amplitudes despite the identical titration points. Using 0.6 units of *E. coli* RNA polymerase, a titration point of 1 μg template implies that there is one initiation site per 1500 DNA base pairs. This corresponds to the number of initia-

tion sites on total DNA as determined by the sucrose gradient centrifugation technique.

It should be noted that the titration curves are multiphasic. This may be due to the presence of several types of RNA polymerase initiation sites with different affinities for the enzyme. Since the maximum activity, obtained in large excess of template, would include all types, the highest titration point was used as an indication of the number of initiation sites.

In order to rule out the possibility that these results were due to the non-specificity of the bacterial enzyme, we did the same experiment using calf thymus RNA polymerase B. Using this enzyme as probe open DNA and covered DNA also had the same number of initiation sites and this was equal to the number of sites found on total DNA (Fig. 2). As was the case with the bacterial enzyme the deter-

Figure 2. Titration of calf thymus RNA polymerase with open and covered regions of calf thymus chromatin. Increasing amounts of sonicated calf thymus DNA (□), open region DNA (○) or covered region DNA (Δ) were added to 0.14 units of RNA polymerase. Incubation conditions were the same as those described in Materials and Methods. After 20 min of propagation in high salt the tubes were assayed for incorporation into trichloroacetic acid-precipitable material. The data are expressed as pmol UTP incorporated in 20 min. Incorporation of UTP in the absence of template was less than 0.5 pmol.

mination of initiation sites was not affected by the size of the DNA. Sonicated DNA (average size 500 base pairs) had the same number of initiation sites as DNA of molecular weight 10^7 daltons. We could not determine the absolute number of initiation sites for the eukaryotic enzyme since the enzyme preparation was not purified to homogeneity and we could not determine the maximum specific activity.

DISCUSSION

Observations of the specific synthesis of globin RNA by avian reticulocyte chromatin in vitro using mammalian erythroid chromatin (1, 3, 19) suggests that the transcriptional controls operating in vivo are preserved in our chromatin preparations and can be recognized by a bacterial polymerase as well as the eukaryotic enzyme. It is becoming increasingly clear from reconstitution experiments (4, 17) that those factors responsible for the specific enhancement of globin RNA synthesis reside within the protein fraction and in particular in the non-histone proteins (5, 6).

The number of RNA polymerase initiation sites on chromatin is preserved after reconstitution (Table 1). Histones, purified from calf thymus chromatin by acid extraction, were also capable of restricting template activity. Thus histones alone can account for the restriction of initiation sites on chromatin. We find that the mode of addition of histones to DNA is important for preserving template activity. When histones were reconstituted with DNA using stepwise salt-urea dialysis, the template activity was similar to that of chromatin. When histones were added directly to DNA, template activity was not restricted (unpublished results). It should be noted that the addition of histones by reconstitution allows histones to redistribute upon the DNA under more nearly equilibrium conditions. This may account for the differences observed using these techniques. Since restricted template activity can be obtained by reconstituting DNA and histones alone, we can assume that the non-histone proteins are probably not essential for the inhibition of template activity. In addition, since chromatin proteins are randomly distributed after reconstitution (20, 21) we can conclude that the specific localization of proteins on the DNA is not a requirement for the restriction of template activity. If the non histone proteins serve as regulatory factors in gene expression, it is possible that they exert their effect by activating specific initiation sites which would

otherwise be restricted by the histones.

In our experiments we find that the exclusion of histone fl results in a large increase in the number of available initiation sites. This is consistent with the early work of Koslov and Georgiev (10) and the results of Felsenfeld *et al.* (17), showing that the removal of histone F2c from native duck reticulocyte chromatin, by methods which do not cause protein rearrangement, causes a 10-fold increase in the number of initiation sites.

*In previous studies of the accessibility of the DNA in chromatin to a variety of chemical and biological probes, it was found that about half of the DNA in chromatin is susceptible either to attack by Micrococcal nuclease or to titration by the polycation, poly-D-lysine (18). These studies demonstrate that the DNA of chromatin can be structurally divided into two distinct classes: one which is tightly bound to protein (covered DNA) and another which is either free or associated with chromatin protein in such a way as to be chemically reactive (open DNA). Both of these fractions can be prepared in the absence of protein or PDL sliding (20). Our experimental methods do not presently permit us to distinguish between the protective effects of histone and non-histone proteins. Most of the covered DNA certainly arises from interactions with histones, which constitute the major protein fraction (75-85%) of our chromatin preparations.

Our experiments indicate that the initiation sites for both *E. coli* RNA polymerase and calf thymus RNA polymerase B are equally distributed in both the open and covered DNA fractions. Covered regions probably represent those portions of the DNA which are tightly bound to proteins. If histones restrict template activity or chromatin by binding and protecting initiation sites, one would expect that most of these sites will be found in the covered regions. Since this is not the case, it suggests that the restriction of template activity is accomplished by a more complicated mechanism probably involving changes in the tertiary structure of the chromatin.

ACKNOWLEDGEMENTS

We would like to thank Dr. Gary Felsenfeld and Dr. Barbara Sollner-Webb for their advice and encouragement and for providing us with their data before publi-

cation. We also want to thank Dr. M. Bustin for providing us with purified calf thymus histones. This project was supported by U.S. Public Health Service Grant No. GM 20483.

REFERENCES

1. Axel, R., Cedar, H. and Felsenfeld, G. (1973) *Proc. Natl. Acad. Sci. U.S.A.* 70, 2029-2032.
2. Axel, R., Cedar, H. and Felsenfeld, G. (1973) *Cold Spring Harbor Symp. Quant. Biol.* 38, 773-783.
3. Gilmour, R.S. and Paul, J. (1973) *Proc. Natl. Acad. Sci. U.S.A.* 70, 3440-3442.
4. Paul, J., Gilmour, R.S., Affara, N., Birnie, G., Harrison, P., Hell, A., Humphries, S., Windass, J. and Young, B. (1973) *Cold Spring Harbor Symp. Quant. Biol.* 38, 885-890.
5. Barrett, T., Maryanka, D., Hamlyn, P.H. and Gould, H.J. (1974) *Proc. Natl. Sci. U.S.A.* 71, 5057-5061.
6. Stein, G., Park, W., Thrall, C., Mans, R. and Stein, J. (1975) *Nature* 257, 764-767.
7. Axel, R., Cedar, H. and Felsenfeld, G. (1975) *Biochemistry* 14, 2489-2495.
8. Cedar, H. and Felsenfeld, G. (1973) *J. Mol. Biol.* 77, 237-254.
9. Bonner, J., Dahmus, M.E., Fambrough, D., Huang, R.C.C., Marushige, K. and Tuan, D.Y.H. (1968) *Science* 159, 47-56.
10. Koslov, Yu. V. and Georgiev, G.P. (1970) *Nature (london)* 228, 245-247.
11. Panyim, S., Bilek, D. and Chalkley, R. (1971) *J. Biol. Chem.* 246, 4206-4215.
12. Berg, D., Barrett, K. and Chamberlin, M. (1971) *Methods in Enzymol.* 21, 506-519.
13. Keding, C., Gissinger, F., Gniazdowski, M., Mandel, J. and Chambon, P. (1972) *Eur. J. Biochem.* 28, 269-276.
14. Keding, C. and Chambon, P. (1972) *Eur. J. Biochem.* 28, 283-290.
15. Hyman, R.W. and Davidson, N. (1970) *J. Mol.* 50, 421-438.
16. Cedar, H. (1975) *J. Mol. Biol.* 95, 257-269.

17. Felsenfeld, G., Axel, R., Cedar, H. and Sollner-Webb, B. (1975) The Structure and Function of Chromatin, Ciba Foundation Symposium 28, 29-41.
18. Clark, R.J. and Felsenfeld, G. (1971) Nature New Biol. 229, 101-106.
19. Steggle, A.W., Wilson, G., Kantor, J., Picciano, O. Falney, A. and Anderson, W.F. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 1219-1223.
20. Bloch, S. and Cedar, H. (1976) Nucleic Acids Res. 3, 1507-1519
21. Zimmerman, S.B. and Levin, C.J. (1975) Biochemistry 14, 1671-1677.