Influence of Chromatin Folding on Transcription Initiation and Elongation by RNA Polymerase III†

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ABSTRACT: Nucleosomes were assembled onto either closed circular plasmids containing a single *Xenopus* 5S RNA gene or a linear tandemly repeated array of *Lytechinus* 5S RNA genes. Both chromatin templates were found to vary in their extent of compaction, depending upon the type and concentration of cation in solution. Compaction of these chromatin templates led to a significant inhibition of both transcription initiation and elongation by RNA polymerase III. Thus, the transcriptional repression observed after incorporation of genes into chromatin depends not only on occlusion of the promoter elements through direct contact with histones but also on compaction of nucleosomal arrays which occurs under the conditions of the transcription reactions.

The organization of DNA into chromatin may influence transcription of genes at several different levels. At the level of transcription initiation, the wrapping of DNA around the histone octamer core to form nucleosomes can prevent the recognition of particular DNA sequences by transcription factors [see Felsenfeld (1992)]. Likewise, the folding of arrays of nucleosomes into compacted structures might further hinder transcription factor access to extensive regions of nucleosomal DNA (Wolffe, 1990). Once transcription is initiated, the elongating RNA polymerase still has to traverse individual nucleosomes and perhaps a chromatin fiber as it progresses down the double helix (Ericsson et al., 1989). Either individual nucleosomes or the chromatin fiber could provide obstacles that might significantly reduce the efficiency of the transcription process.

Inspite of these apparent impediments to the processivity of RNA polymerase, several studies have clearly demonstrated the continued association of DNA with histones during transcription by RNA polymerase II in vivo (Nacheva et al., 1989; Chen et al., 1990; Ericsson et al., 1990). More importantly, several aspects of normal nucleosomal structure appear to be retained during the transcription process (Pederson & Morse, 1990; Felts et al., 1990). How the RNA polymerase moves through an ordered histone–DNA complex is unknown [see Morse (1992) and van Holde et al. (1992)]. In vitro studies have demonstrated that bacteriophage RNA polymerases will progress through one or two nucleosomes without hindrance (Lorch et al., 1987, 1988; Losa & Brown, 1987; Wolffe & Drew, 1989). However, although RNA polymerase II will elongate efficiently through a single nucleosome (Lorch et al., 1987), the polymerase will not efficiently elongate through an array of nucleosomes (Izban & Luse, 1991). Results for RNA polymerase III are more ambiguous; Morse (1989) suggested that a single nucleosome will inhibit transcriptional elongation whereas Felts et al. (1990) suggested that an extended array of nucleosomes was required for significant inhibition of transcriptional elongation. In any case, the observed repression generally has been attributed to properties of individual nucleosome(s).

However, a parameter which has not been studied thoroughly in any of the previous transcription studies is chromatin folding. An array of nucleosome cores represents a dynamic macromolecular complex which can undergo a number of different folding transitions [see Hansen and Ausio (1992)]. In particular, it is becoming increasingly clear that the levels of compaction of chromatin observed in the absence of linker histones result from local nucleosome–nucleosome interactions [Hansen et al., 1989; Yao et al., 1991; see Hansen and Ausio (1992)]. On the basis of these observations, one would predict that the chromatin templates used in the previous transcription studies were present in compacted structures during the transcription reaction. If this is the case, it is possible that nucleosome–nucleosome interactions also contributed to the observed transcriptional repression.

In previous studies, we have investigated the organization of *Lytechinus* and *Xenopus* 5S RNA genes in the nucleosome (Dong et al., 1990; Hayes et al., 1990, 1991; Hansen et al., 1991), the salt-dependent folding of arrays of nucleosomes positioned on 5S RNA genes (Hansen et al., 1989), and the transcription of 5S DNA when incorporated into nucleosomes (Clark & Wolffe, 1991). In this paper, we have examined the influence of chromatin folding on transcription initiation and elongation by RNA polymerase III. We find that compaction of nucleosome arrays occurs under conditions commonly used for in vitro transcription assays. Chromatin compaction contributes significantly to the observed chromatin-dependent inhibition of transcription initiation and elongation by RNA polymerase III.

EXPERIMENTAL PROCEDURES

Materials. Whole chicken blood was obtained from Pel-Freeze (Little Rock, AK) and used as the source of histone octamers. Female *Xenopus laevis* were obtained from Xenopus I (Ann Arbor, MI). Plasmid DNAs and the 208-12 DNA template were purified as described previously (Hansen et al., 1989). The plasmids pXP10 containing the *Xenopus borealis* 5S RNA gene, pXbs Δ3′ + 97, and tDNA^{met} have been previously described (Bogenhagen & Brown, 1981; Clark-

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son et al., 1978; Wolffe et al., 1986). All chemicals were of reagent grade. Radioisotopes were obtained from NEN (Dupont, DE).

Preparation of Histone Octamers. Histone octamers were obtained from chicken erythrocyte nuclei essentially as described (Hansen et al., 1989). Briefly, nuclei prepared from 200 mL of whole chicken blood were digested lightly with micrococcal nuclease (14 units/mg of DNA), lysed in 0.25 mM ethylenediaminetetraacetic acid (EDTA),[1] and centrifuged at 6900*g* for 20 min. The pelleted chromatin was resuspended in 10 mM Tris-HCl/0.25 mM EDTA (pH 7.8) containing 0.35 M NaCl and incubated with carboxymethyl-Sephadex (30 μg/mL) for 3 h at 4 °C to remove histones H1/H5. The resulting stripped chromatin was concentrated to ~30 mg/mL, brought to 2.2 M NaCl/0.1 M sodium phosphate (pH 6.7), and chromatographed on a hydroxylapatite column equilibrated with the same buffer to yield purified histone octamers (Simon & Felsenfeld, 1979). The histone octamers used in these studies contained ≪1% H1/H5, as judged by the complete absence of these linker histones on heavily overloaded SDS gels stained with Coomassie Blue.

Chromatin Reconstitution. In most cases, chromatin was reconstituted from purified histone octamers isolated from chicken erythrocyte nuclei and cloned DNA by a modification of the salt dialysis methods described by Hansen et al. (1989, 1991). Various molar ratios (r)[1] of histone octamers and DNA were mixed initially in 2 M NaCl and transferred to Spectrapor 2 dialysis membranes. The DNA concentration was 50–100 μg/mL. The mixtures were dialyzed for >12 h into 10 mM Tris-HCl/0.25 mM EDTA, pH 7.8 (TE), buffer containing 1 M NaCl, followed by dialysis into TE/0.75 M NaCl for >4 h, and then a final dialysis into TE buffer for >12 h. In some cases, nucleosomes were reconstituted from purified chicken erythrocyte core histones and DNA as previously described. Briefly, DNA and purified core histones were mixed in a solution volume of 400 μL at a DNA concentration of 80 μg/mL (or higher) and with histones at the desired mass ratio to DNA. The buffer was 2 M NaCl in TEM-10 buffer (10 mM Tris-HCl, pH 8.0, 1 mM Na-EDTA, and 10 mM 2-mercaptoethanol). The mixing was done in a siliconized tube and the mixture kept on ice until transfer to the dialysis bag. Dialysis is initially overnight in 500 mL of 5 M urea, 2 M NaCl, and TEM-10 and then in solutions of progressively lower NaCl concentration (1.2, 1.0, 0.8, and 0.6 M) for 80 min each. Dialysis continues for 3–4 h in 1 L of 0.6 M NaCl, 10 mM Tris-HCl, pH 8.0, and 1 mM Na-EDTA with 1 mM 2-mercaptoethanol, and finally the reconstitute is dialyzed overnight against 1 L of 10 mM Tris-HCl, pH 8.0, and 1 mM Na-EDTA with 1 mM 2-mercaptoethanol (Camerini-Otero et al., 1976; Simon & Felsenfeld, 1979; Clark & Felsenfeld, 1991). The properties of chromatin reconstituted by either methodology were identical.

Boundary Sedimentation. Boundary sedimentation velocity studies were carried out in a Beckman Model E analytical ultracentrifuge equipped with scanner optics. Samples were sedimented at 18 000–28 000 rpm using 12-mm double-sector cells and a four-hole AN-F rotor. The temperature was between 18 and 20 °C, and was controlled to within ±0.1 °C. The A_{260} of the samples was between 0.6 and 0.8. Under these conditions, there is a ≤5% concentration dependence of chromatin sedimentation coefficients (J. C. Hansen, unpublished results).

The analog data obtained from the photomultiplier tube were converted to digital form by a CROM-1 A/D board (Metrabyte Corp.), using an interface obtained from Baker Labs (Corvallis, OR). The digitized scans were analyzed by the method of van Holde and Weischet (1978) to obtain the integral distribution of sedimentation coefficients present in the sample, using the Ultrascan data analysis software program (Computer Applications, Missoula, MT). Data were corrected to $s_{20,w}$ in the usual manner using values of 0.55 and 0.65 for the partial specific volume of the DNA and saturated chromatin templates, respectively. The intermediate partial specific volumes of the subsaturated reconstitutes were estimated from the fractional saturation of the templates.

Transcription Buffers. The buffers used for the transcription studies are a modification of the original J buffer described previously (Birkenmeier et al., 1978; Wolffe, 1989a), the major difference being in the amount of $MgCl_2$ present. All transcription buffers contained 10 mM Na-Hepes (pH 7.5), 2 mM dithiothreitol, 0.1 mM Na-EDTA, 50 mM KCl, 0.5 mM each of ATP, GTP, CTP, and UTP, and 5% glycerol. "Low Mg^{2+} transcription buffer" contained each of these components plus 2 mM $MgCl_2$. On the basis of a K_a of $\sim 5 \times 10^{-5}$ M for the ATP–Mg^{2+} interaction (Dawson et al., 1969), the free Mg^{2+} concentration in low-Mg^{2+} transcription buffer is calculated to be <0.1 mM. The "high Mg^{2+} transcription buffer" used in some studies contained the same core components, but with 7 mM $MgCl_2$. After binding of Mg^{2+} by the 2 mM NTPs, the free Mg^{2+} concentration in high-Mg^{2+} transcription buffer is calculated to be 5 mM.

In Vitro Transcription. Extracts of oocyte nuclei were prepared from ovaries of *X. laevis* as described (Birkenmeier et al., 1978; Wolffe, 1989a) except that the collection buffer was low-Mg^{2+} transcription buffer without added NTPs. Transcription reactions were carried out by initially incubating 150 ng of DNA in 20 μL of low-Mg^{2+} transcription buffer with 15 μL of oocyte nuclear extract which had been supplemented with 0.5 mM each NTP. After 30-min incubation, the reaction mixture was diluted to 200 μL with low-Mg^{2+} transcription buffer, 50 μCi of [α-^{32}P]GTP was added, and the incubation was continued for a further 10 min. Importantly, the concentrations of $MgCl_2$ and NTPs remain constant throughout the entire transcription assay. Where indicated in the text, the same sequence of reactions was carried out in high-Mg^{2+} transcription buffer. Transcription products were extracted and analyzed on 7% polyacrylamide gels containing 7 M urea (Wolffe et al., 1986).

RESULTS

Compaction of Circular Chromatin in Low-Mg^{2+} and High-Mg^{2+} Buffer Conditions. We have previously studied the transcriptional activity of small (3.3 kb) circular plasmid DNA molecules containing *Xenopus* 5S rRNA genes which had been reconstituted into chromatin (Clark & Wolffe, 1991). Transcription was found to be independent of superhelical density, but was progressively inhibited by the loading of nucleosomes onto the DNA template. The basis for this inhibitory effect has remained obscure. In particular, it is not known whether the observed inhibition of transcription occurs at the level of nucleosome–promoter interactions, is due to properties unique to an array of nucleosomes such as chromatin folding, or is due to some combination of both. It is not even clear if small circular chromatin templates are capable of compaction. As a result, we first chracterized the structural

[1] Abbreviation: r, moles of histone octamer per mole of 208 bp DNA; TE, 10 mM Tris-HCl/0.25 mM EDTA (pH 8.0); NTPs, nucleoside triphosphates; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

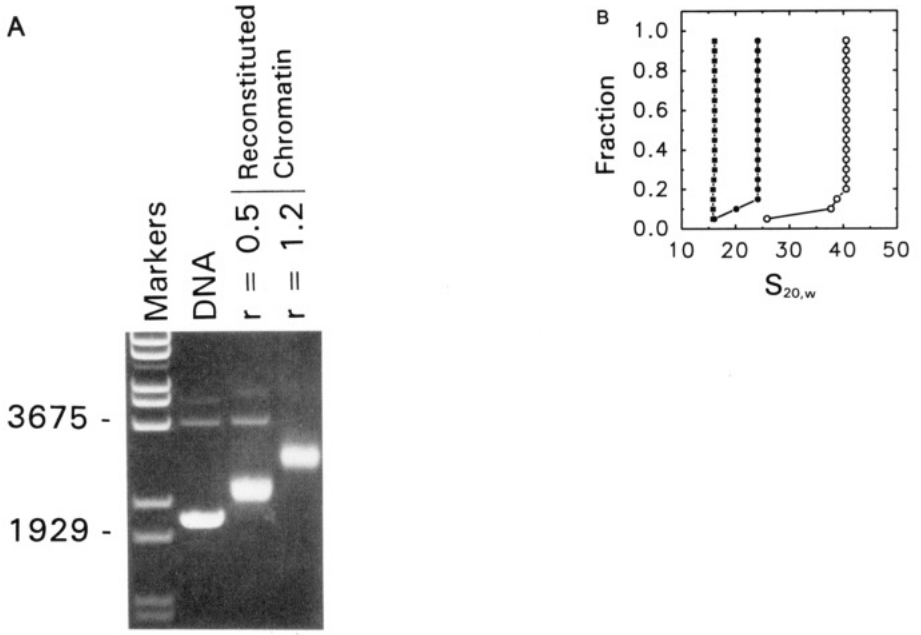

FIGURE 1: Reconstitution of circular pXP10 chromatin. pXP10 plasmid DNA was reconstituted by salt dialysis as described under Experimental Procedures, using molar histone/DNA ratios (r) of 0.5 and 1.2. (A) Agarose gel electrophoresis. Reconstitutes and naked DNA were electrophoresed on a 1.0% agarose gel buffered with 40 mM Tris–acetate/1 mM EDTA at 5 V/cm. The gel was stained with ethidium bromide and visualized under UV illumination. (B) Sedimentation velocity. The $r = 0.5$ (●) and $r = 1.2$ (○) reconstitutes shown in panel A, together with the free pXP10 DNA (■), were sedimented in TE buffer in the analytical ultracentrifuge at 24 000 rpm. The scans of the boundaries were analyzed by the method of van Holde and Weischet (1978) to yield the integral distribution of sedimentation coefficients. The data are plotted as the fraction of molecules (y axis) with an $s_{20,w}$ less than or equal to the value shown on the x axis.

features of circular chromatin templates under the ionic conditions present in the subsequent transcription experiments.

Nucleosomes were reconstituted at molar histone/DNA ratios (r) of 0.5 and 1.2 onto a preparation of circular pXP10 DNA which was ~90% negatively supercoiled and ~10% open circular (nicked). Analysis of these reconstitutes by native agarose gel electrophoresis indicates a progressive retardation of the negatively supercoiled DNA molecules with increasing values of r (Figure 1A). It is important to note that the $r = 0.5$ reconstitutes migrated as a distinct band, with only a barely discernible smearing present between the supercoiled DNA and $r = 1.2$ chromatin end points. Interestingly, at the subsaturating histone/DNA ratio ($r = 0.5$), the nucleosomes assembled quantitatively onto the negatively supercoiled DNA, even though it was present in a 10-fold excess over the relaxed open circular DNA. It has previously been demonstrated that the committed step in nucleosome assembly during the salt dialysis reconstitution method used here is formation of H3/H4 tetramer–DNA complexes (Hansen et al., 1991). Thus, consistent with the results of Clark and Felsenfeld (1991), the data in Figure 1A demonstrate the marked preference of the H3/H4 tetramer for negatively supercoiled DNA.

The presence of nucleosomes on these reconstitutes was verified by micrococcal nuclease protection. Linking number changes indicated that the pXP10 DNA reconstituted at $r = 0.5$ and 1.2 yielded molecules with an average of 10 and 20 nucleosomes per DNA molecule, respectively (data not shown; Clark & Wolffe, 1991). Consistent with previous observations, the nucleosomes on the $r = 1.2$ reconstitutes are spaced every 155–165 base pairs; i.e., they are "close packed" (data not shown; see Figure 4C).

Sedimentation velocity analysis of the free and reconstituted pXP10 templates in TE buffer is shown in Figure 1B. The free DNA molecules sediment between 15.8 and 16.1 S. In the case of the $r = 0.5$ reconstitutes, ~90% of the material sediments at 24 S while ~10% of the material remains at 16 S, confirming that nucleosomes assembled quantitatively onto the supercoiled DNA. In the case of the highly saturated $r = 1.2$ reconstitutes, 90% of the molecules sedimented at 40 S, while 10% sedimented at 30 S. This is consistent with an increased number of nucleosomes present on both the closed circular (40 S) and open circular (30 S) templates. The observed homogeneity in chromatin sedimentation coefficients is in excellent accord with the distinct bands and absence of smearing observed on the agarose gels (Figure 1A). Thus, the sedimentation and electrophoresis results both indicate that reconstitution of these circular DNA templates by salt dialysis is not a positively cooperative process.[2] While the structure of the circular chromatin molecules in TE buffer cannot be deduced directly from the sedimentation data, it has been demonstrated previously that under these ionic conditions, linear chromatin sediments in its maximally extended conformation (Hansen et al., 1989). Similarly, the electron microscopy studies of Simpson et al. (1985) provide support for an extended structure of circular chromatin molecules in solutions of very low ionic strength.

We next wanted to determine whether the circular chromatin templates become compacted under the ionic conditions present during in vitro transcription experiments. As established previously for linear systems (Butler & Thomas, 1980; Hansen et al., 1989), chromatin compaction is indicated by an increased sedimentation coefficient in salt relative to the sedimentation coefficient observed in very low salt buffers. Our standard low-Mg^{2+} transcription buffer contains 50 mM KCl + 2 mM $MgCl_2$ + 2 mM NTPs. Thus, the most obvious

[2] A positive cooperative assembly mechanism would be indicated by the presence of a mixture of entirely naked supercoiled DNA molecules and completely saturated nucleosomal templates at subsaturating values of r. Both the electrophoresis results and the sedimentation velocity results indicate that for subsaturated $r = 0.5$ pXP10 reconstitutes this is not the case. Instead, each DNA template has an intermediate number of nucleosomes present after reconstitution with intermediate molar histone/DNA ratios.

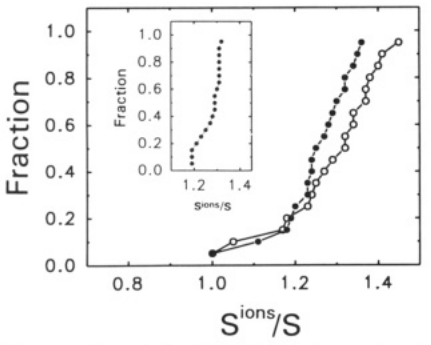

FIGURE 2: Compaction of circular pXP10 chromatin. The $r = 0.5$ pXP10 reconstitutes shown in Figure 1 were sedimented in TE buffer containing 50 mM KCl ("low Mg^{2+} conditions") (●) or 50 mM KCl + 5 mM $MgCl_2$ ("high Mg^{2+} conditions") (○). Scans of the boundaries were analyzed by the method of van Holde and Weischet (1978) to yield the integral distribution of sedimentation coefficients. For each boundary division, data are expressed as the $s_{20,w}$ obtained in the presence of ions divided by the $s_{20,w}$ observed in TE buffer; the latter is obtained from Figure 1B. The ratio expected if no chromatin compaction occurred would be 1.0. Shown in the inset is the $r = 1.2$ chromatin sedimented in the presence of TE + 50 mM KCl. This chromatin aggregated extensively in the presence of 5 mM $MgCl_2$, as judged by the complete absence of absorbance at 260 nm in the supernatant after 60 s of centrifugation at 14 000 rpm in an Eppendorf microfuge.

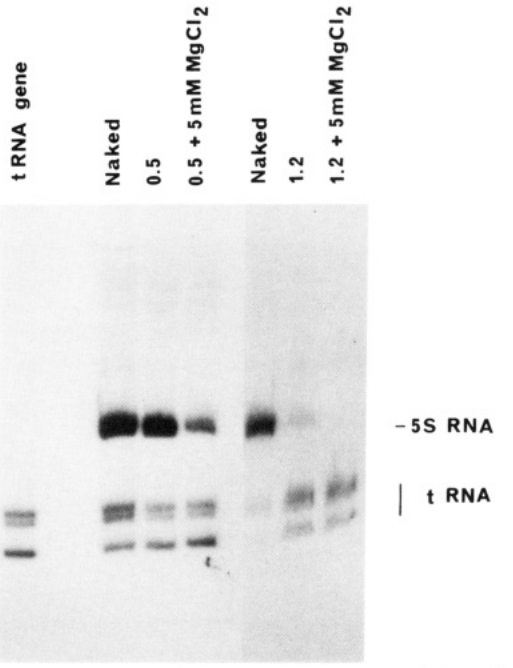

FIGURE 3: Transcription of 5S RNA gene chromatin (pXP10) under different conditions compared to a naked tRNA gene. pXP10 reconstitutes ($r = 0.5$ or $r = 1.2$ as indicated) shown in Figure 1 were transcribed in J buffer or in either low-Mg^{2+} or high-Mg^{2+} transcription buffer (+5 mM $MgCl_2$) as described under Experimental Procedures. Radiolabeled RNAs derived from the templates as indicated were resolved on a polyacrylamide gel, and an autoradiograph was obtained. A naked plasmid containing a tRNA gene ($tDNA^{met}$) was used as an internal control.

experiment would be to determine the chromatin sedimentation coefficients in the absence and presence of 50 mM KCl + 2 mM $MgCl_2$ + 2 mM NTPs. However, this was not possible because of NTP interference with UV scanning. We therefore performed an experiment in which 2 mM NTP was replaced with 2 mM tripolyphosphate (which has a very similar binding constant for Mg^{2+}, but does not absorb light at 260 nm). Results indicate that the extent of folding of defined chromatin molecules in 50 mM KCl + 2 mM $MgCl_2$ + 2 mM tripolyphosphate was not significantly different from the extent seen in 50 mM KCl alone (data not shown). Similarly, there was no significant difference in the extent of folding present in 50 mM KCl + 0.125 mM $MgCl_2$ (the calculated free [Mg^{2+}] in low-Mg^{2+} transcription buffer is <0.1 mM). From these experiments, we conclude that the degree of chromatin compaction observed in buffers containing 50 mM KCl accurately mimics the degree of folding present in low-Mg^{2+} transcription buffer. It also follows that a mixture of 50 mM KCl + 5 mM $MgCl_2$ will accurately mimic the ionic composition of high-Mg^{2+} transcription buffer (which contains 50 mM KCl + 7 mM $MgCl_2$ + 2 mM NTPs).

The sedimentation coefficient profiles of $r = 0.5$ and $r = 1.2$ pXP10 reconstitutes in TE buffer containing 50 mM KCl are shown in Figure 2. At both extents of reconstitution, there is a salt-dependent increase in chromatin $s_{20,w}$. The 20–35% increases in pXP10 sedimentation coefficients in 50 mM KCl are very similar to those observed previously for linear oligonucleosomes in 40 mM NaCl (Hansen et al., 1989). These data indicate that circular chromatin molecules consisting of two different nucleosome densities compact significantly under the ionic conditions associated with low-Mg^{2+} transcription buffer. Importantly, at 50 mM KCl, there is no indication of the oligonucleosome dissociation that occurs at higher monovalent cation concentrations (Hansen et al., 1989, 1991).

We have also investigated the structure of the pXP10 chromatin molecules in the presence of 50 mM KCl + 5 mM $MgCl_2$. These high-Mg^{2+} conditions are similar to the ionic conditions used in most in vitro transcription reactions (Birkenmeier et al., 1978; Dignam et al., 1983; Parker & Topol, 1984; Kamakaka et al., 1991). For the subsaturated $r = 0.5$ reconstitutes, addition of 5 mM $MgCl_2$ leads to a modest increase in the chromatin $s_{20,w}$ relative to the values obtained in 50 mM KCl alone (Figure 2). This indicates that ~60% of the subsaturated closed circular chromatin molecules compact even further in the presence of 5 mM $MgCl_2$. In contrast, the close-packed $r = 1.2$ chromatin molecules form very large aggregates (precipitate) in 5 mM $MgCl_2$. Ausio et al. (1986) have previously reported precipitation of linear fragments of H1-depleted chromatin under high-Mg^{2+} conditions. The same compaction and aggregation behavior of the $r = 0.5$ and 1.2 pXP10 chromatin molecules is observed when the TE buffer is replaced with the 10 mM Hepes (pH 7.4), 2 mM DTT, and 5% glycerol buffer used in the transcription studies (data not shown).

Initiation of Transcription from Circular Chromatin Templates under Conditions of Chromatin Compaction and Aggregation. In the experiments shown in Figure 3, we compared the initiation of transcription from circular pXP10 chromatin templates in low-Mg^{2+} and high-Mg^{2+} transcription buffers. Recall that in simulated low-Mg^{2+} conditions, both the $r = 0.5$ and $r = 1.2$ templates are significantly compacted due to the presence of 50 mM KCl (Figure 2). Addition of 5 mM Mg^{2+} (high-Mg^{2+} conditions) leads to further compaction of the $r = 0.5$ chromatin and aggregation of the $r = 1.2$ chromatin. Thus, by comparing the extent of transcription initiation observed in low-Mg^{2+} versus high-Mg^{2+} conditions, we can determined qualitatively how compaction (in the case of the $r = 0.5$ chromatin) and aggregation (in the case of the $r = 1.2$ chromatin) influence transcription initiation.

In low-Mg^{2+} transcription buffer, there is a reduction of transcription of the chromatin templates relative to the levels observed for naked DNA, especially at a high nucleosome density (Figure 3). This is the classical observation of transcriptional repression after nucleosome assembly onto 5S

genes. Under these ionic conditions, both the saturated and subsaturated chromatin templates are compacted; however, the extent of chromatin compaction is similar for the $r = 0.5$ and $r = 1.2$ reconstitutes (Figure 2). Thus, these results suggest that most of the observed decrease in transcription initiation on $r = 1.2$ templates compared to $r = 0.5$ templates must be due to increased occupancy of the 5S RNA gene promoters by nucleosomes [confirmed by Eco RV protection; see Clark and Wolffe (1991)]. It should be noted that transcription does not decrease linearly as more nucleosomes are assembled onto the template; this is probably due to the fewer remaining free 5S RNA genes competing more effectively for a large excess of transcription factors (Wolffe et al., 1986; Clark & Wolffe, 1991).

Control experiments with naked tRNA gene templates indicate that the transcription of free DNA is essentially the same in low-Mg^{2+} and high-Mg^{2+} transcription buffers. The chromatin, however, behaves differently; when compared to the levels observed in low-Mg^{2+} buffer, transcription on both the $r = 0.5$ and $r = 1.2$ chromatin templates in high-Mg^{2+} buffer (+5 mM $MgCl_2$) is reduced significantly (Figure 3). With the template assembled at low nucleosome density, transcription in high [Mg^{2+}] is reduced to 25% of that at low Mg^{2+} concentration, while at high nucleosome density transcription is eliminated completely. These additional reductions in the levels of transcription initiation in the high-Mg^{2+} transcription buffer cannot be due to increased nucleosome occupancy of promoters. Rather, together with the results shown in Figure 2, we interpret the data to suggest that the Mg^{2+}-dependent transcriptional inhibition of templates with low nucleosome density ($r = 0.5$) results from Mg^{2+}-dependent increases in chromatin compaction while that observed at high nucleosome density ($r = 1.2$) is due to Mg^{2+}-induced chromatin aggregation.

Together, the results shown in Figures 2 and 3 indicate that multiple phenomena simultaneously contribute to the reduced levels of transcription initiation by RNA polymerase III from circular pXP10 chromatin templates: nucleosome occupancy of 5S RNA gene promoters, chromatin compaction, and in some cases chromatin aggregation.

Transcription through an Extended Array of Nucleosomes on Small Circular Templates by RNA Polymerase III. RNA polymerase III requires a complex of proteins to be assembled onto a class III gene before it can initiate transcription and begin to elongate along the double helix (Wolffe & Brown, 1988). The experiments described above (Figure 3) examine the capacity of a transcription preinitiation complex to form on a 5S RNA gene, and of RNA polymerase III to recognize this complex. Local nucleosome–nucleosome interactions clearly influence the transcription initiation process. It is also quite possible that they may influence the elongation of RNA polymerase III. The remaining sections of this paper test this possibility, first looking at elongation through small circular chromatin templates similar to those used above, followed by studies with more well-defined linear templates.

RNA polymerase III will terminate transcription when it reaches a DNA sequence consisting of three or more T residues flanked by G and C residues (Bogenhagen & Brown, 1981; Cozzarelli et al., 1983). Normally, transcription by RNA polymerase III is efficiently terminated at the end of a 5S RNA gene at the second T of a 5′-GCTTTTGC-3′ DNA sequence. If this sequence is removed, the RNA polymerase will continue to transcribe until it reaches a similar sequence (Bogenhagen et al., 1980; Bogenhagen & Brown, 1981).

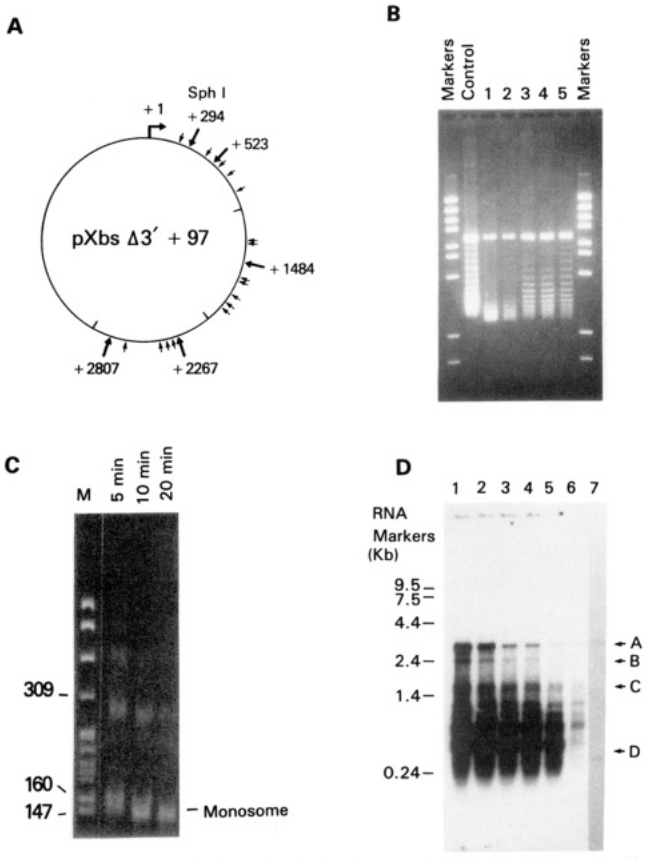

FIGURE 4: Transcription through chromatin assembled on small circular plasmids by RNA polymerase III. (A) Schematic representation of plasmid pXbs Δ3′ + 97, showing transcription termination sites for RNA polymerase III. The plasmid pXbs Δ3′ + 97 is shown with the start site of 5S RNA gene transcription indicated as +1. There is an *Sph*I restriction endonuclease site indicated by the large arrow (+294). The number next to the large arrows is the number of base pairs 3′ of the start site for transcription. The other large arrows indicate major predicated sites for transcription termination (see text) whereas the small arrows are minor sites for termination. (B) Estimation of nucleosomes on reconstitutes. The reconstitutes (lanes 2–5) or naked DNA (lane 1) was relaxed with nicking closing extracts deproteinized, and then topoisomers were resolved on a 1% agarose gel containing chloroquine (30 μg/mL). Chloroquine was removed and the gel stained with ethidium bromide before photography. Markers are of λDNA digested with *Bst*EII. (C) The reconstitute shown in panel B, lane 5, was digested with micrococcal nuclease for the times indicated before resolution on a 6% nondenaturing polyacrylamide gel. Markers of pBR322 DNA digested with *Msp*I are shown (markers). The gel was stained with ethidium bromide, and the photograph is shown. (D) Transcripts from pXbs Δ3′ + 97 as naked DNA, or reconstituted with different numbers of nucleosomes, in low-Mg^{2+} transcription buffer (lanes 1–6) or high-Mg^{2+} transcription buffer (lane 7). Radioactive transcripts are resolved on a 1.5% agarose gel after glyoxalation; RNA size markers (BRL) are indicated. The letters A, B, C, and D correspond to the major predicted sites of transcription termination (at 2807, 2267, 1484, and 523, respectively); other observed sites are discussed in the text. Lanes 1 and 6 correspond to two different masses of naked DNA being transcribed (100 and 5 ng). Lanes 2–5 show the transcription of 100 ng of plasmid DNA reconstituted with 2–7 nucleosomes on average. Lane 7 shows the transcription of plasmid DNA reconstituted with 6–7 nucleosomes in high-Mg^{2+} transcription buffer.

In the construct pXbs Δ3′ + 97 shown in Figure 4A, the terminator sequence of the *Xenopus* 5S RNA gene has been removed and the gene fused to pBR322 DNA (Bogenhagen & Brown, 1981). RNA polymerase III is predicted to stop transcription both at weak terminators (three "T"s, small arrows) and at strong terminators (four or more "T"s, large arrows). Also marked (with a large arrow) is a restriction endonuclease (*Sph*I) site 294 bp downstream of the start of

transcription (hooked arrow). This site is predicted to be incorporated into the first nucleosome that might be downstream of the transcription complex assembled on the *Xenopus* 5S RNA gene. The transcription complex includes protein–DNA interactions over 150 bp downstream of the start of transcription (Wolffe & Morse, 1990). Nucleosomes were assembled onto plasmid pXbs Δ3′ + 97 by the method of Clark and Wolffe (1991), and the extent of chromatin assembly in this case was measured by relaxing the reconstitutes with topoisomerase I followed by resolution of the deproteinized DNA on agarose gels containing chloroquine (Figure 4B). The formation of nucleosomes was assayed by micrococcal nuclease digestion (Figure 4C). The average number of nucleosomes reconstituted onto the template was 4–5 for samples 3 and 4 and 6–7 for sample 5. The plasmid pXbs Δ3′ + 97 is 4173 bp in length; therefore, samples 3 and 4 have approximately one nucleosome every 640 bp. This average distribution does not imply that some nucleosomes cannot pack closely together [see Clark and Wolffe (1991)].

Transcription of the naked DNA and reconstituted chromatin templates shown in Figure 4A–C was carried out for 30 min at 30 °C in low- or high-Mg^{2+} transcription buffer. Quantitation of radioactive RNA precursors incorporated into newly synthesized RNA revealed that overall transcription was reduced as increasing levels of nucleosomes were reconstituted onto the plasmid. This reduction in net transcription has two origins. The first involves decreased transcription initiation due to increased promoter occupancy and chromatin compaction (Figure 3). The second involves a reduction in the capacity of RNA polymerase III to elongate along the nucleosomal template (Figure 4D).

Transcription of naked plasmid pXbs Δ3′ + 97 reveals the distribution of transcript lengths expected from the analysis shown in Figure 4A. The transcripts labeled A–D correspond within 10% to the lengths of the major termination sites predicted. The three transcripts between C and D correspond to use of the minor predicted termination sites in this region. As increasing numbers of nucleosomes are assembled onto the template and transcribed at low Mg^{2+} concentrations, the size distribution of the transcripts clearly changes such that the longer ones disappear and the shorter transcripts remain. Those sites closest to the initiation site of transcription are utilized most frequently (Figure 4A). For comparison, radioactive products of a separate transcription reaction in low-Mg^{2+} transcription buffer (Figure 4D, lane 6) using less naked DNA as a template were loaded adjacent to the transcription reaction using the reconstitute containing six to seven nucleosomes (Figure 4D, lane 5). The change toward generating shorter transcripts when nucleosomes are reconstituted onto the template is clearly seen. Under high-Mg^{2+} buffer conditions (Figure 4D, lane 7), transcription is very severely inhibited compared either to naked DNA (lane 1) or to a chromatin template reconstituted to the same extent but transcribed under low-Mg^{2+} buffer conditions (lane 6). Control experiments indicated that both long and short transcripts were stable in the extract.

The results in Figure 4D suggest that RNA polymerase III has difficulty in elongating through a nucleosomal template, in agreement with Felts et al. (1990) and Morse (1989). However, under low-Mg^{2+} buffer conditions, transcripts >1000 bp in length are obtained before transcription elongation is severely diminished. These transcripts are significantly longer than might be expected if RNA polymerase could not elongate through nucleosomes at all, especially for the templates containing an average of 1 nucleosome every 640 bp (considering that each nucleosome minimally contains 146 bp of DNA). Under high-Mg^{2+} buffer conditions, transcription by RNA polymerase III through a chromatin template appears to be very inefficient [see also Morse (1989)].

An independent approach to the question of whether RNA polymerase III can elongate through more than a single nucleosome makes use of the ability of nucleosomes to inhibit restriction enzymes from cleaving DNA that is incorporated into a nucleosome (Lorch et al., 1987; Morse, 1989). As shown in Figure 5A, if the position occupied by a reconstituted nucleosome includes the *Sph*I restriction enzyme cleavage site, the chromatin template will remain intact in the presence of *Sph*I. In contrast, if a nucleosome is *not* present on the *Sph*I site, the chromatin molecule will be linearized. If RNA polymerase III can elongate through the nucleosome incorporating the *Sph*I site, when the reconstitute remains intact, the pattern of transcription observed should include transcripts terminating at the sites in the vector as described for Figure 4A. If the reconstitute is linearized, run-off transcripts should be observed (Morse, 1989). At the low levels of reconstitution used in these experiments, we will expect to have a mixture of linearized and intact templates in the reaction mixture. Southern hybridization of the gel shown in Figure 4C confirmed that the *Sph*I site was incorporated into nucleosomes.

Plasmid pXbs Δ3′ + 97 was reconstituted with an average of six to seven nucleosomes per molecule ($r = 0.4$). The reconstitute was then incubated in oocyte nuclear extract at 30 °C under low-Mg^{2+} buffer conditions and in the presence of the restriction endonuclease *Sph*I for varying periods of time. After 10 min, naked DNA was completely digested by *Sph*I (Figure 5C, lane 3). In contrast, the reconstitute was only partially digested (Figure 5C, lane 2), and over the following 110 min, very little additional digestion occurred (Figure 5C, lane 1). From this experiment, we conclude that a nucleosome assembled so as to include the *Sph*I site will prevent *Sph*I from restricting the DNA.

Digestion of pXbs 3′ + 97 chromatin by *Sph*I leads to a mixed population of molecules (Figure 5C). Where the *Sph*I site is incorporated into the nucleosome, the template remains intact; where it is not, the template is linearized. Transcripts from naked DNA incubated for 120 min under low-Mg^{2+} transcription buffer conditions at 30 °C in the oocyte nuclear extract, and to which the radioactive precursor [α-^{32}P]GTP was added for the final 10 min, are shown resolved on a 6% denaturing polyacrylamide gel (Figure 5B, lane 1). The length of the smallest major transcript in this lane is approximately 520 nucleotides, suggesting that it represents termination at the first major termination site at +523 (Figure 4A). In the presence of *Sph*I, the major transcript observed is the expected "run off" transcript of approximately 300 nucleotides. A second major transcript has a length of approximately 240 nucleotides and probably corresponds to termination at a run of 3 "T" residues upstream of the *Sph*I site (Figure 4A). We conclude that under these conditions RNA polymerase III can elongate through a nucleosome. If the nucleosome had prevented transcriptional elongation by RNA polymerase, we might have expected either a "road block" at which a single predominant truncated transcript would appear (this would depend upon a nucleosome being translationally positioned over the *Sph*I site) or a distribution of truncated transcripts corresponding to randomly positioned nucleosomes blocking polymerase. In each case, novel transcripts should appear that are visible in the reconstitute, but not in naked DNA. Such transcripts are not observed (Figure 5B, compare lanes

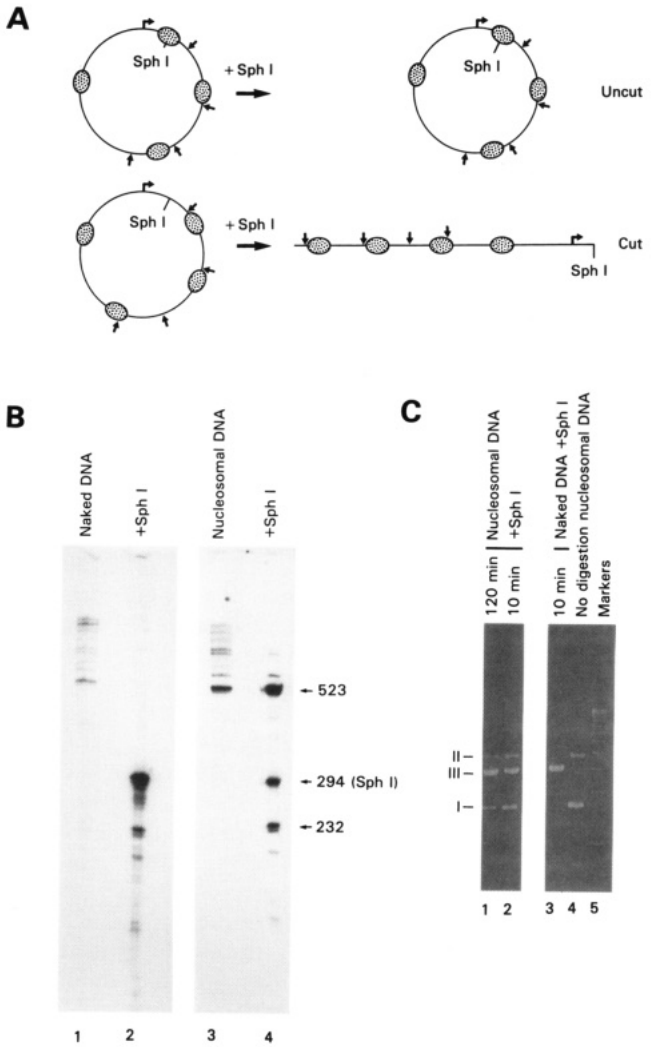

FIGURE 5: Transcription through a nucleosome by RNA polymerase III. (A) Experimental rationale. Nucleosomes are positioned randomly on the plasmid DNA. Two possibilities are shown: when a nucleosome is positioned on the *Sph*I site, the site cannot be restricted; hence, the plasmid DNA will remain a closed circle; when a nucleosome is not positioned on the *Sph*I site, the enzyme can cleave the DNA. When the plasmid reconstituted with nucleosomes is uncut, the capacity of RNA polymerase III to transcribe through a nucleosome can be assessed by examining the frequency with which different termination sites can be used (arrows at 90° to circle) downstream of the start site of transcription (hooked arrow). When the *Sph*I site is cut, RNA polymerase III will generate "run-off" transcripts at this site. In practice, the results include a mixture of these two possibilities (see text for details). (B) RNA polymerase III transcribes through a nucleosome immediately 3′ of the transcription complex. Nucleosomes were reconstituted (6–7/plasmid molecule on average) onto pXbs Δ3′ + 97. One hundred nanograms of naked DNA or reconstitute (as indicated) was transcribed in low-Mg^{2+} transcription buffer alone (lanes 1 and 3, respectively) or plus 10 units of *Sph*I (BRL) (lanes 2 and 4). After 90 min, radioactive precursor ([α-^{32}P]GTP) was added and the reaction continued for a further 30 min. The radioactive transcripts were purified, resolved on a 6% polyacrylamide gel containing 7 M urea, and radioautographed. (C) Digestion of naked DNA and reconstitutes in oocyte nuclear extract. Naked DNA (lane 3) or nucleosomal DNA (lanes 1 and 2) was incubated under transcription conditions in oocyte nuclear extract plus *Sph*I for the time indicated before DNA was extracted, deproteinized, and resolved on a 1% agarose gel. A photograph of the gel stained with ethidium bromide is shown; input nucleosomal DNA without digestion (lane 4) and a 1-kb ladder purchased from BRL (lane 5) are shown for reference. The positions of supercoiled (I), relaxed closed circular (II), and linear DNA (III) are indicated.

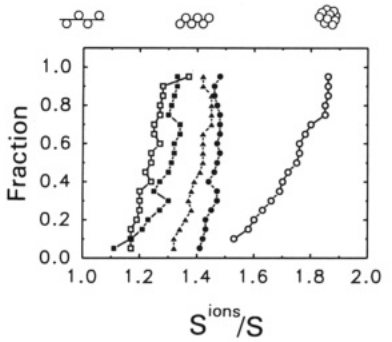

FIGURE 6: Transcription of 208-12 oligonucleosomes under conditions of chromatin compaction. Compaction of 208-12 oligonucleosomes in simulated low-Mg^{2+} and high-Mg^{2+} transcription buffers. Linear 208-12 DNA was reconstituted at r = 0.3 (□), 0.6 (■), 0.9 (▲), and 1.2 (●) by the method of salt dialysis. Sedimentation velocity analysis in TE buffer (not shown) indicated a homogeneous distribution of reconstitutes at each of the r values (as in Figure 1B). The r = 0.3, 0.6, 0.9, and 1.2 reconstitutes sedimented at 13.0, 17.3, 21.0, and 28.7 S, respectively. These reconstitutes were sedimented in core transcription buffer [10 mM Hepes (pH 7.4), 2 mM DTT, 0.1 mM EDTA, and 5% glycerol], followed by sedimentation in the same buffer containing 50 mM KCl. In the case of the r = 1.2 reconstitutes, the sample was also sedimented in core transcription buffer containing 50 mM KCl + 5 mM $MgCl_2$ (○). For each boundary fraction, data are expressed as the sedimentation coefficient obtained in the presence of transcription buffer + ions divided by the sedimentation coefficient obtained in core transcription buffer alone (s^{ions}/s). The predicted sedimentation coefficients of the beads-on-a-string, contacting zig-zag, and solenoidal conformations of the 208-12 chromatin molecule are 28, 39, and 52 S, respectively (Hansen et al., 1989). Thus, the zig-zag structure would be expected to sediment 1.40 times faster, and the solenoid structure 1.85 times faster, than the extended structure known to be favored in the absence of salt (Hansen et al., 1989). This is illustrated schematically at the top of Figure 6.

2 and 4). In experiments such as this, one might be concerned about the possibility of nucleosome sliding. Sliding of nucleosomes in vitro is associated with much higher levels of monovalent cations than the 50 mM KCl used in these experiments [see van Holde (1988)]. In addition, conditions that favor sliding also lead to nucleosome dissociation. Such dissociation, which has been observed from chromatin templates at >100 mM NaCl (Hansen et al., 1989, 1991), is not detected in either the low-Mg^{2+} or the high-Mg^{2+} buffer used here (Figures 2 and 6). Thus, it is very unlikely that nucleosome sliding is occurring in these experiments.

It is important to note that the efficiency of transcription on the small closed circular templates used in Figures 4 and 5 was highly dependent on the free Mg^{2+} concentration in the buffer. Compared to low-Mg^{2+} conditions, in high-Mg^{2+} transcription buffer transcriptional elongation by RNA polymerase III was further reduced (Figure 4D, compare lanes 5 and 7; data not shown) to the extent reported previously by Morse (1989). This suggests that nucleosome–nucleosome interactions, and the resulting compaction of the nucleosome array, might be responsible for much of the reduced processivity of RNA polymerase III through a nucleosomal array.

The circular chromatin templates used above are not well-defined. As a result, at this point we are only able to make qualitative statements regarding the influence of chromatin compaction on transcription elongation. In order to further address this issue, we turned to a novel DNA template which was equally well suited for quantitation of both transcription elongation and chromatin folding.

Transcription through a Linear Array of Positioned Nucleosomes under Defined Conditions of Chromatin Compaction. A potential means of obtaining homogeneous preparations of well-defined chromatin templates capable of supporting RNA polymerase III-dependent transcription was developed by Simpson et al. (1985), who engineered DNA molecules consisting of 12 tandem repeats of a DNA sequence

which contains the nucleosome positioning signal of the *Lytechinus* 5S RNA gene (Simpson & Stafford, 1983). This DNA we term the 208-12 template. Upon reconstitution with histone octamers, these constructs yield defined-length oligonucleosomes comprised of positioned nucleosomes separated by linkers (Simpson et al., 1985; Hansen et al., 1989, 1991; Dong et al., 1990; Meersseman et al., 1991). Importantly, each 208-12 repeat contains the sequences necessary for transcription initiation by RNA polymerase III, but lacks the termination sequences. Upon initiation of transcription at any 1 of the 12 repeats, RNA polymerase III will proceed along the double helix until it reaches the end of the DNA molecule, or until it is blocked. Furthermore, the sedimentation coefficients of a number of different extended and folded conformations of the 208-12 chromatin template have been accurately predicted and experimentally verified (Hansen et al., 1989). Thus, this represents an ideal synthetic chromatin template to explore the effects of chromatin compaction on polymerase processivity.

In order to obtain nucleosome arrays consisting of different levels of template saturation, the 208-12 DNA template was reconstituted at various molar histone/DNA ratios by salt dialysis. Previous work has established that saturated 208-12 dodecanucleosomes sediment in TE buffer at 29 S (Hansen et al., 1989). In these studies, we obtained saturated (i.e., 29 S) dodecanucleosomes at $r = 1.2$. Similar to the case observed for the pXP10 template (Figure 1), the 208-12 DNA template reconstituted at $r = 0.3$, 0.6, and 0.9 yields narrow distributions of subsaturated reconstitutes (not shown). On the basis of the incremental change in sedimentation coefficient which occurs with increasing extents of template saturation,[3] the $r = 0.3$, 0.6, and 0.9 reconstitutes are estimated to contain 2–4, 5–7, and 8–10 nucleosomes per DNA template, respectively. Importantly, the sedimentation analysis detects no evidence of even small amounts of naked 208-12 DNA (i.e., 11S-sedimenting material) at any of these subsaturating ratios. Similar estimates are obtained from analysis of the ratio of free 5S repeats and chromatin fragments produced after digestion with *Eco*RI. The nucleosomes present on the highly subsaturated 208-12 templates are distributed randomly among the 12 binding sites present on each strand; there is no evidence for large amounts of nucleosome clustering.[3] Thus, even the $r = 0.3$ and $r = 0.6$ oligonucleosomes are unusually well-defined chromatin molecules, containing ~700 and ~250 bp linkers, respectively.

Sedimentation velocity analysis of the 208-12 reconstitutes indicates that there is a progressive increase in the sedimentation coefficient change in 50 mM KCl as the nucleosome density increases from 2–3/template (20–30% increase) to 12/template (45% increase) (Figure 6). In the case of the saturated dodecanucleosome arrays ($r = 1.2$), a mixture of 50 mM KCl and 5 mM $MgCl_2$ leads to a much greater extent of compaction than is observed in 50 mM KCl alone. Interestingly, unlike the more poorly defined $r = 1.15$ pXP10 reconstitutes in which nucleosomes are close-packed, the spaced arrays under high-Mg^{2+} conditions do not aggregate.

There is an excellent correlation between the observed salt-dependent changes in oligonucleosome sedimentation coefficients and the values predicted (Hansen et al., 1989) upon folding of the extended 208-12 oligonucleosome into more compact conformations. The observed 40% increase in the chromatin sedimentation coefficient in the presence of 50 mM KCl is consistent with compaction of the extended dodecanucleosomes into an intermediate contacting zig-zag structure (Figure 6). The maximum extent of compaction observed in Figure 6 is similar to that observed previously in comparable TE/NaCl conditions (Hansen et al., 1989).

In the presence of 50 mM KCl + 5 mM $MgCl_2$, the saturated $r = 1.2$ 208-12 chromatin exhibits 55–85% increases in $s_{20,w}$. This is a much greater increase in sedimentation velocity than has been observed in monovalent cations alone (Figure 6; Hansen et al., 1989, 1991). Interestingly, an 85% increase in $s_{20,w}$ is expected upon folding of an extended 208-12 oligonucleosome molecule into a higher order solenoidal structure (Hansen et al., 1989). This suggests that under these high-Mg^{2+} conditions, some of the saturated reconstitutes are folding even further into a highly condensed globular conformation (Figure 6). The additional changes in sedimentation coefficient caused by the 5 mM Mg^{2+} are associated with profound effects on the transcriptional activity of the 208-12 chromatin template (see below).

We next examined the transcriptional properties of these same 208-12 oligonucleosomes under the various conditions of chromatin compaction observed in Figure 6. Recall that the principle of this experiment was to make use of the "terminator-less" *Lytechinus* 5S RNA gene fragment (Simpson et al., 1985) that retained the necessary promoter elements for transcription initiation (like pXbs $\Delta 3' + 97$) but lacked a termination signal. The intact *Lytechinus* 5S RNA gene is active in *Xenopus* oocyte nuclear extract under low-Mg^{2+} conditions (Figure 7A, lane 3). When the 208-12 DNA is used as a template, a series of large (up to 2500 bp) transcripts are synthesized. There are designated read-through transcripts (Figure 7A, lane 2) and result from initiation at 1 of the 12 promoters followed by elongation to the end of the DNA template. These RNA species represent bonafide transcripts initiated from the 5S promoters; no transcription is observed from an analogous template (172-12) composed of 12 tandem repeats of a deletion mutant of *Lytechinus* DNA lacking key internal promoter elements (Figure 7A, lane 1).

Transcription of the various 208-12 reconstitutes in low-Mg^{2+} transcription buffer is shown in Figure 7A. The striking observation that transcripts greater than 1500 bp are generated from even the mostly saturated $r = 0.9$ and completely saturated $r = 1.2$ chromatin templates clearly indicates that RNA polymerase III is capable of elongating through multiple nucleosomes. This result is not due to loss of nucleosomal structure in the transcription extract; *Eco*RI digestion of the chromatin ($r = 0.9$) before and after transcription releases single repeats of the 5S RNA gene incorporated into nucleosomes, whose mobilities and level of assembly before and after transcription on nondenaturing polyacrylamide gels are identical (Figure 7B, compare lanes 2 and 3). As observed previously with the small circular chromatin templates (Figures 3 and 4), assembly of nucleosomes onto the 5S RNA genes does, however, reduce the levels of both transcription initiation and polymerase elongation along the DNA. That is, as the level of reconstitution increases, both the total number and the average length of the transcripts decline (Figure 7A, lanes 4–7) relative to the control. Included in these samples as an internal control is a naked plasmid containing an intact *Lytechinus* 5S RNA gene. This generates a 120-nucleotide transcript (Figure 7A, marked as 5S RNA). The amount of this transcript remains almost constant in all cases. Quantitative analysis reveals a 10-fold decline in the overall transcription of the $r = 0.9$ chromatin relative to the naked control template (not shown).

[3] J. C. Hansen and D. Lohr, submitted.

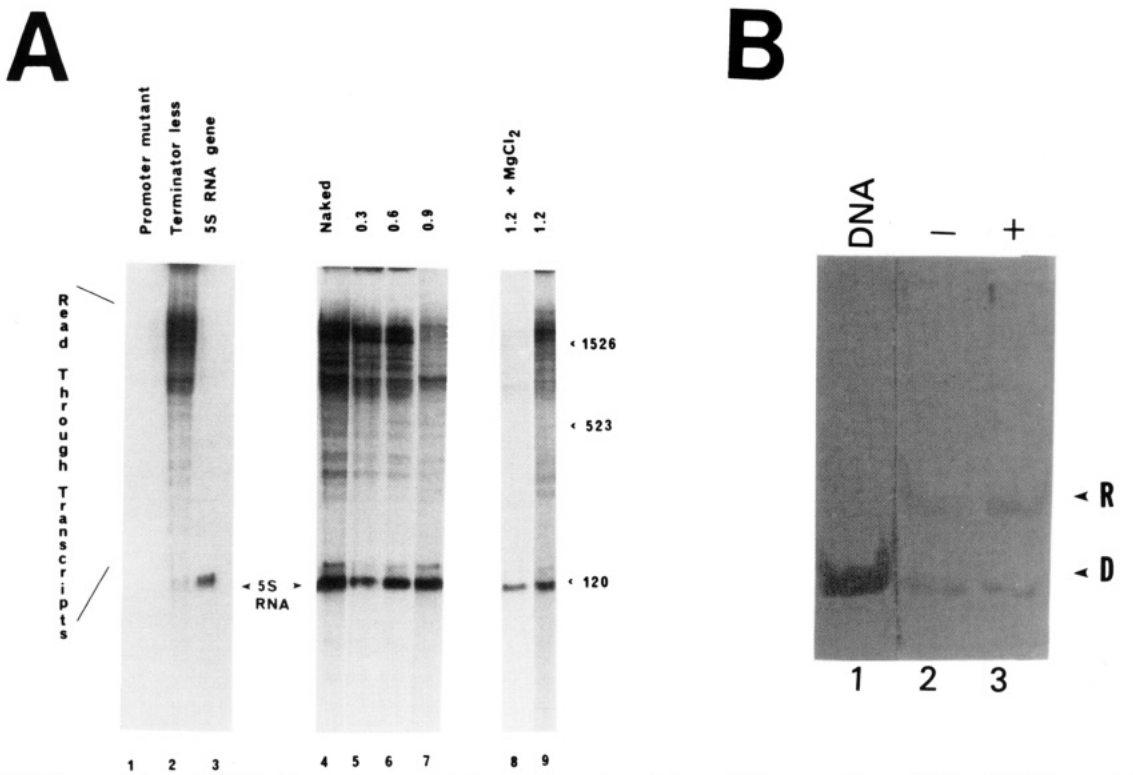

FIGURE 7: (A) Transcription of 208-12 oligonucleosomes. The reactions shown in lanes 1–7 were performed in low-Mg^{2+} transcription buffer as described in the text. Shown are transcripts derived from the 172-12 template promoter mutant (lane 1), the tandemly repeated terminatorless 208-12 DNA template (lane 2), and the intact *Lytechinus* 5S RNA gene (lane 3). The position of intact 5S RNA (120 nucleotides) is indicated. Lanes 4–7 show transcription of naked 208-12 template DNA, or of this template progressively reconstituted into chromatin as indicated. Lanes 8 and 9 compare transcription of the fully loaded templates ($r = 1.2$) in low-Mg^{2+} versus high-Mg^{2+} transcription buffers. Size markers and read-through transcripts are indicated. (B) Nucleosome integrity following transcription. The chromatin samples ($r = 0.9$) transcribed in Figure 7A, lane 7, were treated with *Eco*RI before (–) or after (+) transcription. *Eco*RI digestion releases single repeats of the 5S RNA gene incorporated into nucleosomes. These are then resolved on a 4% nondenaturing acrylamide gel and stained with ethidium bromide. The relative proportions of nucleosomal structures (R) to free DNA (D) remain unchanged after transcription. Free DNA repeats are used as markers (DNA). A negative image of the stained gel is shown to enhance definition.

Both the nucleosome occupancy of 5S gene promoters and the degree of chromatin compaction in low-Mg^{2+} transcription buffer increase progressively with increasing values of r (Figure 6). Thus, it is impossible at this point to separate the inhibitory effects of salt-dependent chromatin compaction from those of promoter occlusion through incorporation into nucleosomes. However, on the basis of the results obtained in Figures 2–5, it is quite likely that both phenomena contribute significantly to the inhibition of transcription in low-Mg^{2+} buffer.

The effects of high-Mg^{2+} conditions on transcription are more clear-cut, and again are striking. Recall that high-Mg^{2+} conditions induced additional increases in compaction of the 208-12 dodecanucleosomes to levels predicted for a solenoid. Concomitantly, the transcriptional activity of the $r = 1.2$, 208-12 chromatin in high-Mg^{2+} transcription buffer is severely reduced (Figure 7A, compare lanes 8 and 9). Importantly, transcription of the naked 5S RNA gene internal control was not significantly different in low-Mg^{2+} versus high-Mg^{2+} transcription buffers. Additional inhibitory effects of high [Mg^{2+}] on transcription of the $r = 0.3$, 0.6, and 0.9 reconstitutes were observed, although they were less dramatic than for $r = 1.2$ chromatin (data not shown). We conclude that the highly condensed solenoid-like structures of the $r = 1.2$ 208-12 chromatin molecules (formed in high-Mg^{2+} conditions) are incapable of supporting significant levels of transcription by RNA polymerase III.

DISCUSSION

It is well documented that assembly of nucleosomes onto DNA templates reduces transcription by RNA polymerases II and III (Figures 3–5 and 7; Wolffe, 1990; Felsenfeld, 1992). This result generally has been interpreted as being due to repression of transcription initiation and (or) elongation by the nucleosome per se. While inhibition at the level of the nucleosome is a contributory factor, our results indicate that the ion-dependent compaction of nucleosomal arrays also reduces the levels of both transcription initiation and elongation by RNA polymerase III. In our experiments, no linker histones are present; chromatin compaction is driven largely by nucleosome–nucleosome interactions [see Hansen and Ausio (1992)]. This implies that local contacts between nucleosomes may be important in determining the accessibility of a promoter to transcription factors and RNA polymerase, as well as the efficiency with which RNA polymerase will progress along the chromatin template. We also observe aggregation of densely loaded chromatin templates in the presence of the high Mg^{2+} concentrations often used in transcription experiments. Chromatin aggregation totally abolishes transcription.

Comparison of our transcription initiation data (Figures 2 and 3) with published work on RNA polymerase III transcription (Shimamura et al., 1988; Morse, 1989; Almouzni et al., 1990, 1991; Felts et al., 1990) suggests that a substantial fraction of the previously observed transcriptional repression of nucleosomal templates is due to chromatin-specific structural changes. A similar conclusion most likely applies to the transcription of nucleosomal templates by RNA polymerase II (Knezetic & Luse, 1986; Workman et al., 1988). It is important to note that the relative contributions to transcriptional repression resulting from individual nucleosomes, chromatin compaction, and chromatin aggregation will be

dependent on the nature of the template, the extent of template saturation, and the monovalent and divalent cation concentrations in the transcription buffer.

Our transcription studies were done at comparable chromatin and ion concentrations as the sedimentation studies; however, the transcription reactions are performed in an oocyte nuclear extract containing MgNTPs. It is possible that the MgNTPs and (or) some component of the nuclear extract may further modify the extent of compaction induced by cations alone. Thus, future work with purified systems will be necessary to precisely quantitate the relationships between chromatin compaction and transcription.

Transcriptional Elongation by RNA Polymerase III through Chromatin. Our results indicate clearly that under certain conditions RNA polymerase III will elongate through nucleosomes (Figures 4, 5, and 7). If only a few nucleosomes are present on a closed circular template, RNA polymerase III will proceed efficiently through at least one nucleosome, but will proceed less efficiently through the remainder of the array (Figures 4). In the case of the linear-positioned 208-12 chromatin, we see full-length transcripts generated from even the saturated arrays, although the trend toward shorter transcript lengths is observed with increasing template saturation. In low-Mg^{2+} conditions, it is not clear to what extent this is due to impedance of the polymerase by the nucleosomes per se, and to what extent it results from the intermediate levels of chromatin compaction that result from ion-dependent nucleosome–nucleosome interactions. However, if a linear array is further compacted into a highly condensed structure (using high-Mg^{2+} conditions), processivity is even more severely inhibited (Figure 7).

Our results are in general agreement with those of Felts et al. (1990) and Morse (1989), who observed a reduced efficiency of transcription by RNA polymerase III through a nucleosomal array. Minor differences in the observed extent of inhibition are probably due to the higher level of free Mg^{2+}, and thus chromatin compaction, in the Morse (1989) experiments. Similar results on nucleosomal arrays have been obtained by Izban and Luse (1991) for RNA polymerase II and by O'Neill et al. (1992) for T7 RNA polymerase. Although in these cases the compaction of chromatin was not examined directly, the ionic conditions used in the experiments will almost certainly have induced compaction of the chromatin templates.

The specificity with which RNA polymerase III terminates transcription along DNA does not change on the reconstituted nucleosomal template as compared to naked DNA (Figure 2D), even though the structure of the DNA changes following its association with the histone octamer (Hayes et al., 1991). It is possible that this may be a consequence of a totally random array of nucleosomes on the plasmid molecule, such that no one barrier to elongation arises (Zimmerman & Levin, 1975; Morse, 1989). We favor an alternative explanation, which is that RNA polymerase III elongates more slowly through a nucleosomal template. This conclusion is based on the observation that termination sites close to the transcription initiation site are used more frequently. The efficiency with which the enzyme recognizes a termination signal (Cozzarelli et al., 1983) may depend on the rate of progression along the DNA, such that a slower elongating enzyme is more likely to terminate at a given termination signal. Precedents for this exist in prokaryotic termination, where regions of dyad symmetry upstream of a termination signal cause the RNA polymerase to slow down and terminate more effectively (Gilbert, 1976; Rosenberg & Court, 1979). Furthermore, it has been shown that the elongation rate of both prokaryotic and eukaryotic RNA polymerases on a chromatin template is less than that observed on naked DNA (Williamson & Felsenfeld, 1978; Wasylyk et al., 1979; Meneguzzi et al., 1979; Wasylyk & Chambon, 1979).

The tendency of RNA polymerase III to recognize a termination site more readily in a nucleosomal template is supported by the maintenance (Figure 4D) or actual increase in the number of shorter transcripts under low-Mg^{2+} transcription buffer conditions. This suggests that the enzyme dissociates at a termination signal and then reinitiates rather than arrests elongation randomly with the transcript still attached. Similar conclusions have recently been described by Izban and Luse (1991) for RNA polymerase II. Our quantitation of transcription initiation events [not shown; see Clark and Wolffe (1991)] indicates that each promoter is utilized over 20 times per hour, even on the reconstitutes with 6–7 nucleosomes per molecule. This observation further supports the idea that the RNA polymerase III molecules terminate, dissociate from the template, and reinitiate transcription. The severe inhibition of transcription observed under high-Mg^{2+} transcription buffer conditions (Figure 4D, lane 7) indicates that divalent cation concentration might severely restrict RNA polymerase dissociation and reutilization in chromatin.

From our studies, it seems likely that both the histone octamer and local nucleosome–nucleosome interactions contribute to a decreased rate of elongation by RNA polymerase III. The transcription complex assembled on a transcriptionally active 5S RNA gene keeps at least 150 bp of DNA free from association with a histone octamer (Wolffe & Morse, 1989). Therefore, after each initiation event, RNA polymerase approaches the *first* nucleosome it sees (including the *Sph*I site in pXbs $\Delta 3' + 97$) from naked DNA. In contrast, the next nucleosome is approached from DNA in some form of association with a histone octamer. Interaction with histones, e.g., the histone tails, or between nucleosomes themselves might restrict processivity. It is interesting to note that elongation of RNA polymerase III through correctly spaced arrays of nucleosomes on the linear *Lytechinus* repeats was found to be more efficient than on the supercoiled DNA molecules containing close-packed nucleosomes (compare Figures 4 and 7). This might reflect either inhibitory effects of interactions between close-packed nucleosomes or a topological restriction to processivity (Clark & Felsenfeld, 1991). It is important to note that reduced rates of elongation attributed to histone–DNA interactions are not likely to explain the reduction of transcription on chromatin compaction seen in Figure 3 since the 5S RNA gene including the terminator of transcription is completely covered by non-histone proteins (Wolffe & Morse, 1990). Modification of nucleosome structure, for example, by acetylation of histone tails (Norton et al., 1989), might facilitate progression of RNA polymerases in vivo [see van Holde et al. (1992) and Morse (1992) for a discussion]. The capacity of RNA polymerase III to elongate through nucleosomes clearly is further reduced by additional folding of the nucleosomal array into a highly compacted solenoidal structure (Figures 6 and 7). This lends support to the idea that the higher order chromatin structure stabilized by linker histones in vivo is a transcriptionally inactive conformation. Future experiments should examine whether our observations with RNA polymerase III can be extended to RNA polymerases I and II and consider the problem of how these RNA polymerases transcribe a true chromatin template.

The Dominant Role of Nucleosome Cores in Chromatin Folding. During the course of this work, we have generated a number of novel results regarding the contributions of nucleosome cores to chromatin folding. Our studies with circular arrays of nucleosome cores indicate that small circular chromatin molecules, like their linear counterparts, are quite capable of undergoing compaction in the presence of monovalent and divalent cations (Figure 2). These results provide physical evidence to support the folding of small circular DNA molecules inferred previously from in vivo work (Thoma & Zatchej, 1988). As has been documented for linear fragments (Yao et al., 1991), the compaction of circular chromatin templates lacking linker histones most probably is caused by intramolecular nucleosome–nucleosome interactions that occur upon screening of the linker DNA charge by cations.

Interestingly, the extent of compaction of both linear and circular chromatin molecules in 50 mM KCl is not strongly dependent on linker DNA length (Figures 2 and 6; Hansen et al., 1989). In the case of linear 208-12 DNA templates which contain only 2–4 nucleosomes/template, the bulk of the linker DNA ranges from 250 to 750 bp, yet this chromatin sediments a full 20–30% faster in the presence of 50 mM KCl (Figure 6; data not shown). In the case of the circular pXP10 template, the maximum increases in sedimentation coefficients of the $r = 0.5$ and $r = 1.2$ reconstitutes in 50 mM KCl were very similar (Figure 2). Again, these results are consistent with a dominant role for local nucleosome–nucleosome interactions in chromatin compaction (Hansen & Ausio, 1992).

Biological Relevance. A large number of experiments have indirectly or directly suggested a role for chromatin folding in the regulation of gene expression in vivo. Histone H1 is required for the repression of oocyte-type *Xenopus* 5S RNA genes in vivo (Schlissel & Brown, 1984; Wolffe, 1989b). Histone H1-mediated chromatin folding appears important for sequestering transcription complexes from RNA polymerase III, and may play a role in displacing transcription factors (Chipev & Wolffe, 1992). Several proteins in addition to histone H1 are postulated to play a role in stabilizing the 30-nm-diameter chromatin fiber, for example, histone H5 in chicken erythrocytes or HP1 in *Drosophila*. Chromatin isolated from chicken erythrocyte nuclei that contains histone H5 also has template-engaged RNA polymerase II present; this enzyme ceased to elongate as chromatin condensed (Hentschel & Tata, 1978). Similarly, the chromatin-associated non-histone protein HP1 is involved in the formation of heterochromatin, and directs the repression of transcription through unknown mechanisms probably involving whole domains of the chromatin fiber (James & Elgin, 1986). Our work with defined chromatin templates provides a biophysical demonstration that chromatin compaction can influence gene transcription by RNA polymerase III, as well as provides a structural framework with which to investigate the molecular bases for these phenomena.

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