Repressive Effect on Oligonucleosome Transcription of the Core Histone Tail Domains†

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ABSTRACT: Histone−DNA templates for bacteriophage T7 RNA polymerase were assembled from histone octamers and three different DNA species, two circular (pGEMEX-1 and pT207-18) and one linear (T7-207-18). pGEMEX is devoid of nucleosome positioning sequences, while in pT207-18 and T7-207-18 the region downstream of the promoter contains 18 tandem repeats of a 207 bp positioning sequence derived from the 5S RNA gene of the sea urchin *Lytechinus variegatus*. Elimination of the histone tails in the assembled oligonucleosomes by trypsin digestion is accompanied, in all three DNA species, by substantial increases in transcription efficiency, assayed at different KCl and $MgCl_2$ concentrations, after allowing for the aggregation observed under certain conditions. In the absence of KCl and at low $MgCl_2$ concentration, the presence of 2 mM spermidine causes substantial aggregation of the intact oligonucleosomes but has a much smaller effect on those trypsin digested. The untreated histone−DNA templates, assembled on pGEMEX-1 and T7-207-18, give transcription products significantly shorter than those obtained with the corresponding free DNA. With oligonucleosome templates lacking histone tails, the transcripts have an average length intermediate between those corresponding to free DNA and intact histone−DNA, which indicates a partial elimination of the elongation restrictions imposed by intact histone octamers. The absence of histone terminal domains facilitates both transcriptional initiation and elongation. Apparently, the interaction of the histone tails with DNA at the nucleosomal level is responsible, at least in part, for their repressive effect on transcription.

The core histone octamer, $(H2A{\cdot}H2B{\cdot}H3{\cdot}H4)_2$, interacts with DNA to form the nucleosomal core, the basic structural unit of chromatin (*1*). Each of the four core histones comprises a structured central domain, an amino-terminal tail, and in some cases a carboxy-terminal tail (H2A and H3) (*1*). The central domains are mainly responsible for the structural organization of the nucleosomal core (*2*−*5*), while the amino-terminal tails, which contain a high proportion of basic amino acid residues, are flexible and highly mobile at moderate salt concentrations (0.3−0.4 M) and bind to negatively charged DNA increasing the thermal stability of the particle (*3*, *6*−*8*). In the crystallographic studies of the histone octamer and the nucleosomal core, the location of the histone tails is not well defined (*1*). Although recent crystallographic and biochemical studies (*9*, *10*) locate part of the core histone tails at low ionic strength, the sites of interaction of most of these regions are not yet known. The histone tail domains contribute to the interaction between adjacent nucleosomal elements and to the folding of nucleosomal strands into higher order structures (*11*, *12*). These structural properties make the tails of core histones particularly suitable to modulate chromatin structure and function. Modification of these protein domains might be the means to promote changes from one functional state to another in vivo (*1*).

A valuable approach to study the relationships between chromatin structure and transcription efficiency is the use of in vitro transcription systems (*13*). However, in addition to the complexity of the eukaryotic systems, which makes necessary the use of crude extracts, the low level of RNA synthesis usually obtained with eukaryotic RNA polymerase II, with only a small fraction of the templates being transcribed, makes it difficult to ascertain the chromatin structure of the transcribed templates. With prokaryotic polymerases, RNA synthesis is very efficient, and most or all of the potentially active templates are transcribed. Despite the important differences between eukaryotic and prokaryotic transcription, the results obtained with both prokaryotic and eukaryotic polymerases detect similar constraints of the nucleosomal structure on transcription initiation and elongation (*14*−*18*). Accordingly, it has been suggested that the mechanism used by prokaryotic and eukaryotic polymerases to transcribe through nucleosomes is likely to be essentially the same (*19*). Independent of the similarities between eukaryotic and prokaryotic transcription, prokaryotic RNA polymerases are extremely useful functional probes, since to achieve RNA synthesis the prokaryotic enzyme must separate the two DNA strands and interact in a processive way along one of them, detecting the constraints derived from the association of DNA with histones and the changes in this association that can facilitate the process.

In vitro transcription experiments with both prokaryotic and eukaryotic RNA polymerases indicate that the presence

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of histone octamers associated with DNA at the promoter blocks transcription by preventing initiation, while their location in the coding region inhibits the elongation of RNA chains (*14*−*18*). Covalent modification of core histones in vivo might modulate their effect on transcription by altering their interaction with DNA. In accordance with this proposal, the physiological acetylation of core histones, which only takes place at their amino-terminal domains, has been associated with a transcriptionally active state of chromatin (*20*−*22*). In a previous work (*23*), it was shown that the transcriptional stimulation obtained upon treatment of nucleosomal cores with acetic anhydride is correlated with the modification of histone amino-terminal domains. Recently, Ura et al. (*24*), studying transcription of a dinucleosome template by RNA polymerase III, have shown that the physiological acetylation of core histones significantly facilitates transcription. These results underline the potential role in transcription of the physiological modification of core histone amino-terminal domains.

The purpose of this work is to investigate, using oligonucleosomal templates and bacteriophage T7 RNA polymerase, the transcriptional effects of the absence of core histone tails. Since the structural differences corresponding to the presence or absence of regularly spaced nucleosomes and topological constraints could affect RNA synthesis, three different DNA templates covering these possibilities were employed. Other important conditions affecting chromatin folding and consequently transcription are the concentrations of monovalent and divalent cations. Accordingly, the effects of different salt conditions on transcription of the employed templates have been evaluated. The results show that elimination of the histone tails of the oligonucleosome templates is accompanied by a significant increase in RNA synthesis under most salt conditions investigated. Both initiation and elongation are facilitated by elimination of the histone tails.

EXPERIMENTAL PROCEDURES

Three different transcription templates for bacteriophage T7 RNA polymerase were employed. Plasmid pGEMEX-1 (4 kb, Promega Biotec) includes one promoter and one terminator region for T7 RNA polymerase, the terminator being separated from the initiation site by a coding region of approximately 1.1 kb devoid of strong nucleosome-positioning sequences. Plasmid pT207-18 (6.5 kb), constructed by O'Neill et al. (*18*), contains downstream from a T7 transcription promoter 18 tandem repeats of a 207 bp strong positioning sequence derived from the sea urchin *Lytechinus variegatus*. T7-207-18 (3.8 kb) is a linear DNA molecule comprising the T7 transcription promoter and the 18 downstream 207 bp tandem repeats of pT207-18. It was excised from pT207-18 by digestion with *Hae*III and *Dde*I, according to O'Neill et al. (*25*), and was separated from smaller fragments by chromatography on Sephacryl S-1000 (*11*). Core histone octamers were prepared from chicken erythrocyte nuclei by extraction with 4 M NaCl after removal of histones H1 and H5 with 0.8 M NaCl (26). H2A·H2B dimers and $(H3{\cdot}H4)_2$ tetramers were isolated from core histone octamers by chromatography on Sephadex G-200 (*27*).

Histone−DNA complexes were assembled from the chosen DNA template and histones by dialysis against decreasing salt concentrations (*28*). DNA (60 μg/mL) was mixed at 4 °C with $(H3{\cdot}H4)_2$ tetramers plus the complementary amount of H2A·H2B dimers in 10 mM Tris-HCl (pH 7.8), 2 M NaCl, and 0.25 mM EDTA. The assembly mixtures were dialyzed against solutions containing decreasing NaCl concentrations (*28*). Trypsin digestion of the assembled oligonucleosomal templates (33 μg of DNA/mL) was conducted at 4 °C for 40 min in 10 mM Tris-HCl (pH 7.8) and 0.25 mM EDTA, with 90 μg of trypsin/mL (from bovine pancreas, Sigma). Digestion was ended by the addition of trypsin inhibitor (from soybean, Sigma) at a final concentration of 0.25 mg/mL, and the preparation was immediately employed in the transcription and solubility assays.

Transcription by bacteriophage T7 RNA polymerase (Boehringer Mannheim) (6.7 units/μL) was estimated by measuring the radioactivity from [^{3}H]UTP incorporated into acid-insoluble RNA. The transcription template (7.5 ng of DNA/μL) was preincubated at 37 °C for 5 min with 0.4 mM each of ATP, CTP, GTP, and UTP (33 nCi of [^{3}H]UTP/μL) in 10−40 mM Tris-HCl (pH 8.0), 0−67 mM KCl, 1.6−6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, and human ribonuclease inhibitor (1 unit/μL). Transcription took place at 37 °C and was initiated by the addition of RNA polymerase. After incubation for 7 min (unless otherwise indicated), transcription was ended by the addition of 1 mL of 10% trichloroacetic acid and 0.3% sodium pyrophosphate and the incubation on ice for 20 min. The mixture was filtered through a Whatman GF/C filter, which was then washed twice with 6 mL of trichloroacetic acid/pyrophosphate solution. The radioactivity in the filter was measured after drying. Transcription assays were done in duplicate, the values reported for RNA synthesis being the average of the two determinations.

To investigate the effects of nucleosomal structures on elongation with pGEMEX-1 templates, transcription was conducted under conditions of low reaction rate. The transcription template (7.5 μg of DNA/mL) was preincubated at 15 °C for 5 min with 40 μM ATP and GTP in 40 mM Tris-HCl (pH 8.0), 7 mM KCl, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, human ribonuclease inhibitor (1 unit/μL), and RNA polymerase. At the end of preincubation, 40 μM UTP and 1 μM [α-^{32}P]CTP were added, and incubation took place at 15 °C for 3 or 6 min. The reaction was ended by addition of 10 mM EDTA (pH 7.4), 100 mM NaCl, and 14 μg of salmon DNA/mL. The extracted RNA was subjected to electrophoresis in 4% polyacrylamide gel containing 8 M urea. The distribution and quantitation of RNA chains along the gel was determined with a phosphorimager (Fujifilm BAS-1.500). Both radioactive and nonradioactive size markers were included in the electrophoresis. The radioactive markers were obtained by transcribing in the presence of [α-^{32}P]CTP mixtures of the digestion products of pGEMEX-1 obtained with each of the three restriction enzymes: *Hind*III, *Acc*I, and *Nhe*I. The corresponding transcripts are 959, 429, and 71 nucleotides long.

Electrophoretic analysis of histones was conducted in a 10−20% linear gradient polyacrylamide gel containing 0.1% sodium dodecyl sulfate (29).

The solubility of the different templates was determined at room temperature by measuring the absorbance at 260

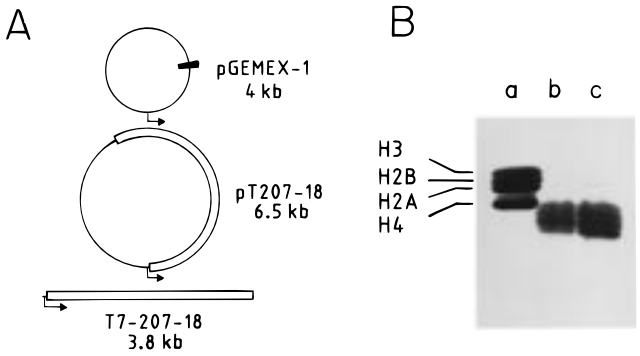

FIGURE 1: Oligonucleosomal components. (A) DNA template molecules. Arrows indicate the location of the initiation site and the sense of transcription for T7 RNA polymerase. The wedge across pGEMEX-1 shows the location of the terminator region. The boxes in pT207-18 and T7-207-18 denote the tandem repeat 207-18. (B) Electrophoresis of the histones obtained from the untreated oligonucleosomes assembled on pT207-18 at a weight ratio of histones to DNA equal to 0.8 (a) and those from two different samples of trypsin-digested templates (b and c).

nm under conditions for high solubility [10 mM Tris-HCl (pH 8.0), 5 mM EDTA] and in the desired solution after separation of the aggregated material by centrifugation (*30*). Solubility is expressed as the percentage of the sample present in the supernatant after centrifugation.

Sedimentation velocity and sedimentation equilibrium experiments were carried out at 20 °C in a Beckman XL-A analytical ultracentrifuge equipped with scanner optics, using 12-mm double sector cells and a four-hole Ti60 rotor. In the sedimentation velocity studies, the samples (A_{260} equal to 0.5) were sedimented at 24 000 rpm. The sedimentation velocity data were analyzed with the program VELGAMMA (supplied by Beckman; see refs *31* and *32*). The sample for sedimentation equilibrium experiment ($A_{260} = 0.2$, 2 mm column height) was centrifuged at 1600 rpm (4 days). Data were collected in the step scan mode set at 0.001 radial increment. Each point was the average of 10 separate absorbance measurements taken at each radial position. The average molecular mass was obtained with the program EQASSOC (supplied by Beckman; see ref *33*) using a specific volume of 0.62, obtained from the values for free DNA and histone octamer (*3*).

RESULTS

Oligonucleosomal structures to be used as transcription templates were assembled on three different DNA molecules: two circular, pGEMEX-1 and pT207-18, and one linear, T7-207-18 (Figure 1A). All three have a promoter for bacteriophage T7 RNA polymerase. With respect to the sequence downstream of this promoter, pGEMEX-1 lacks nucleosomal positioning sequences, while pT207-18 and T7-207-18 show 18 tandem repeats of a 207 bp strong positioning sequence derived from the 5S RNA gene of the sea urchin *Lytechinus variegatus* (*18*, *25*). The use of these DNA templates will allow us to determine the differences, if any, between regular and irregularly spaced nucleosomal structures and between topologically free and constrained templates.

Assembly of oligonucleosomal structures on the three chosen DNA species took place by dialysis against decreasing salt concentrations, using core histone octamers from chicken erythrocytes. With pGEMEX-1, at least 14 histone octamers are assembled on each plasmid at a weight ratio of histones to DNA equal to 1, which gives an average nucleosome density higher than one per 280 bp of DNA (*28*). At the same weight ratio of histones to DNA, approximately an average of one nucleosome is assembled per 220 bp of DNA on pT207-18 (*18*). Nucleosomes assembled on the 5S-207 tandem repeat sequences form regular arrays, with a nucleosomal repeat of 207 bp (*25*). The number of nucleosome positioning sequences occupied by histone octamers in the T7-207-18 template under our experimental conditions (assembly at a weight ratio of core histones to DNA equal to 0.8) was estimated by equilibrium sedimentation. An average molecular mass of 3.97×10^6 was obtained, corresponding to an average occupation of 14 of the 18 positioning sites. An independent evaluation of occupancy made use of the presence of a unique *Dra*I restriction site at nucleotide position 63 in the tandemly repeated sequence, this site being protected from digestion by the assembled histone octamer (*18*). By determining the extent of digestion by *Dra*I of the site nearest the promoter in the employed template as compared to free DNA and to a saturated oligonucleosome, it was estimated that 70% of the sites are occupied by nucleosomes. The studied oligonucleosome has an average sedimentation coefficient of 38 S in 10 mM Tris-HCl (pH 8.0) and 0.25 mM EDTA. This value rises to 44 S in the presence of 0.4 mM $MgCl_2$ and to 50 S in 2 mM. These results indicate that, although the template is not saturated with histone octamers, it is significantly folded by magnesium ions.

To investigate the global transcriptional effect due to the loss of the amino-terminal domains of core histones, oligonucleosomal templates were assembled from each of the three chosen DNA molecules and total core histones, and the assembled oligonucleosomal templates were directly digested with trypsin under conditions causing elimination of the amino-terminal tails (Figure 1B) (*34*). RNA synthesis took place soon after stopping trypsin digestion by the addition of trypsin inhibitior. Relevant controls showed that the presence in the reaction mixtures of trypsin plus its inhibitor has no effect on RNA synthesis by any of the assayed templates.

Hansen and Wolffe (*35*) reported that compaction of chromatin templates led to a significant inhibition of transcriptional initiation and elongation by RNA polymerase III. The extent of chromatin compaction depends on the concentrations of monovalent and divalent cations (*11*, *12*, *30*, *36*−*38*). Since the tails of core histones play a role in the folding of the chromatin fiber, the transcriptional effects caused by removal of these terminal domains were studied under different salt conditions. Figure 2A shows the levels of RNA synthesis obtained with the trypsin-digested and the untreated oligonucleosomal templates, expressed as percentage of the RNA synthesized with the corresponding free DNA template (pGEMEX-1, pT207-18, and T7-207-18). Four salt conditions were employed, corresponding to the combination of moderate concentration (67 mM) or the absence of KCl and high (6 mM) or low (1.6 mM) $MgCl_2$ concentration. With all three free DNA templates (see legend to Figure 2), maximal transcription efficiency is obtained without KCl and with high $MgCl_2$ concentration (condition

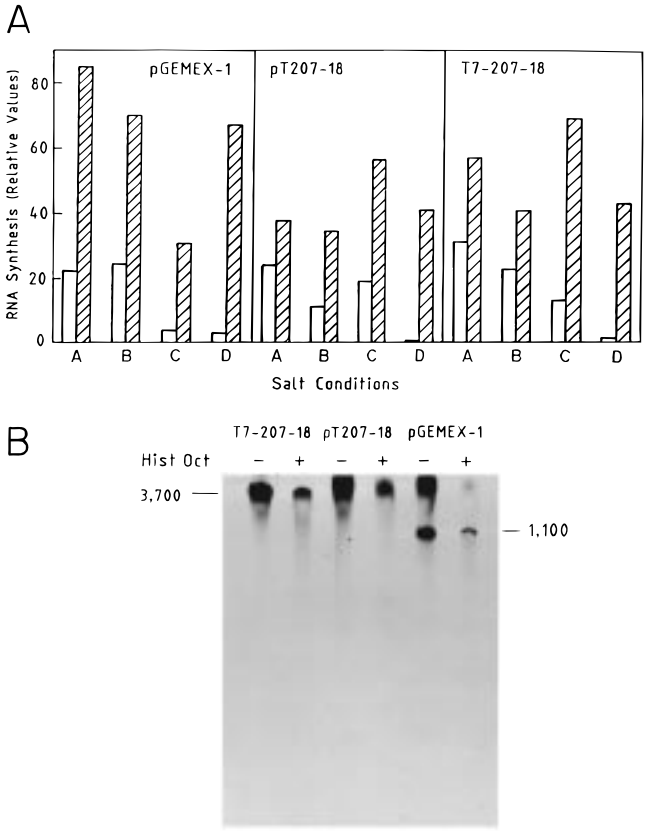

FIGURE 2: Transcription of intact and trypsin-digested oligonucleosomal templates under different salt conditions. (A) RNA synthesis of untreated (open columns) and trypsin-digested oligonucleosome templates (hatched columns) is expressed as percentage of the synthesis obtained with the corresponding naked template under the same assay conditions. Assembly took place at a weight ratio of histones to DNA equal to 1 (pGEMEX-1) and 0.8 (pT207-18 and T7-207-18). Transcription was conducted as indicated under Experimental Procedures with the following variations. KCl concentration: 67 mM (conditions A and B) or 0 mM (conditions C and D). $MgCl_2$ concentration: 6 mM (conditions A and C) or 1.6 mM (conditions B and D). The assay mixture corresponding to salt condition D contained 10 mM Tris-HCl (pH 8.0). With the free DNA templates, the percent values of RNA synthesis relative to that obtained under salt condition C are indicated below. pGEMEX-1: 83 (condition A), 66 (condition B), and 13 (condition D). pT207-18: 69 (condition A), 52 (condition B), and 13 (condition D). T7-207-18: 25 (condition A), 52 (condition B), and 21 (condition D). Under salt condition C, the amount of radioactivity from [^{3}H]UTP incorporated into RNA was similar with the three naked templates: p-GEMEX-1 (107 000 cpm), pT207-18 (104 000 cpm), T7-207-18 (80 000 cpm). (B) Electrophoresis of the transcription products obtained at high KCl and $MgCl_2$ concentrations (condition A). The numbers give the size of the indicated bands in nucleotides.

C). Minimal transcription efficiency of the naked DNA templates (6−14% of that reached under condition C) is obtained in the absence of KCl and with low concentration of free Mg^{2+} (condition D). With all DNA species employed and at the four salt conditions, the RNA synthesis obtained with the intact oligonucleosome templates is substantially increased after trypsin digestion (Figure 2A).

Figure 2B shows the transcription products obtained with the three free DNA templates and the corresponding intact oligonucleosomes, after reaction in the presence of 67 mM KCl and 6 mM $MgCl_2$ (condition A). In all cases, incomplete RNA chains represent a very small part of the transcription products, and they do not appear significantly enriched with oligonucleosome templates as compared with the corresponding free DNA. These results are in agreement with those previously reported for pGEMEX-1 (*27*, *28*, *39*). Since incomplete RNA chains are not accumulated, abortive liberation of transcripts does not appear to be quantitatively important in the transcription of oligonucleosome templates. The employed reaction conditions do not seem to facilitate the study of the effects of nucleosome structures on elongation. With pGEMEX-1 templates, in addition to RNA chains of approximately 1100 nucleotides corresponding to transcription from the promoter to the terminator region, a band of much larger chains is observed. These large products are synthesized when the transcription machinery fails to stop at the terminator region. The presence of histone octamers increases the efficiency of the termination signal in pGEMEX-1, as was previously reported (*27*, *28*, *39*).

Since aggregation of oligonucleosomes by magnesium ions requires the presence of the core histone tails (*30*, *38*, *40*), the observed transcriptional stimulation might be caused, at least in part, by the different aggregation of intact and trypsin-digested templates. To investigate this point, the solubility of the intact oligonucleosome templates was determined under the different salt conditions (Figure 3A). With the three DNA species in the presence of 67 mM KCl, with and without free magnesium ions (conditions A and B), the moderate aggregation level of the intact oligonucleosomes might have only a small effect on the stimulation observed after trypsin digestion. However, in the absence of KCl (conditions C and D), there is a larger decrease in solubility of the different intact oligonucleosomes. When the solubility of the oligonucleosome templates is expressed relative to that of the corresponding free DNA, the values obtained under salt condition C rise to around 80%, and those corresponding to salt condition D rise to 60%. In contrast with this low solubility of the intact oligonucleosome templates under salt condition D, the values obtained with the corresponding trypsin-digested templates are similar to those of free DNA (Figure 3B). Therefore, the different extent of aggregation of intact and trypsin-digested templates appears to be partially responsible for the stimulation observed in the absence of KCl. The decreased solubility of the intact oligonucleosomes in the absence of KCl and at low concentration of $MgCl_2$ was initially unexpected. However, in addition to KCl and $MgCl_2$, 2 mM spermidine was included in the solubility mixtures since it is routinely present in the transcription assays.

To investigate the contribution of spermidine to the observed aggregation, solubility of the three intact oligonucleosomes was determined under the previously employed salt conditions but in the absence of spermidine. The results (Figure 3C) show a large increase in solubility in the absence of KCl (conditions C and D). Without spermidine and at low free magnesium, solubility is close to 100% (conditions B and D), while at high free magnesium (conditions A and C) a small but significant aggregation is observed. Therefore, the presence of spermidine in the absence of KCl and at low free magnesium (condition D, Figure 2A) is responsible for the observed aggregation of the intact oligonucleosomes. The presence of KCl and free magnesium partially prevents aggregation by spermidine (Figure 3A, conditions A−C). Apparently, spermidine is displaced from its interaction with oligonucleosomes by potassium and magnesium ions, which decreases aggregation.

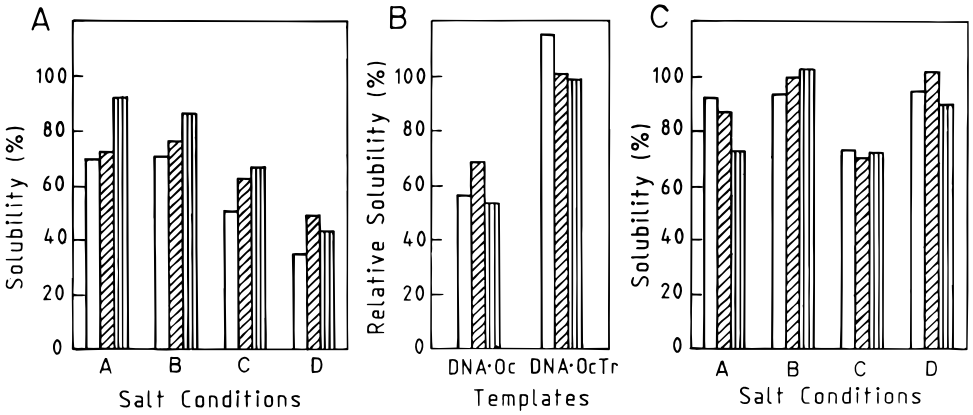

FIGURE 3: Solubility of oligonucleosomal templates under different salt conditions. The open columns correspond to templates containing pGEMEX-1, and the hatched ones correspond to templates with pT207-18 (diagonal hatching) and T7-207-18 (vertical hatching). (A) Solubility of untreated oligonucleosomes in the presence of 2 mM spermidine. Assembly took place at a weight ratio of histones to DNA equal to 1 (pGEMEX-1) and 0.8 (pT207-18 and T7-207-18). The assay mixtures were as those employed for transcription (Figure 2) but without NTPs, RNA polymerase, and ribonuclease inhibitor and with the following $MgCl_2$ concentrations: 4.4 mM (conditions A and C) and 0.2 mM (conditions B and D). The $MgCl_2$ concentrations were modified relative to those employed in the transcription experiment (Figure 2) to compensate for the magnesium ions bound to NTPs. (B) Solubility of intact and trypsin-digested oligonucleosomes in the presence of 2 mM spermidine and salt condition D, relative to the corresponding free DNA. The solubility is expressed as the percentage of soluble template relative to the corresponding free DNA soluble under the same conditions. (C) Solubility of untreated oligonucleosomes in the absence of spermidine. As in the experiment of panel A but with spermidine eliminated from the mixtures.

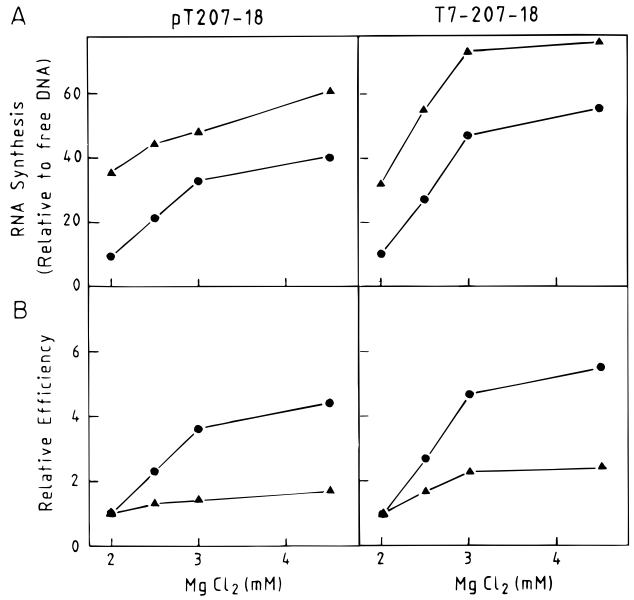

FIGURE 4: Dependence on $MgCl_2$ concentration of the RNA synthesis obtained with intact and trypsin-digested templates in the absence of KCl and spermidine. (A) RNA synthesis, of untreated (●) and trypsin-digested oligonucleosome templates (▲) obtained with pT207-18 and T7-207-18 (as indicated), is expressed as percentage of the synthesis obtained with the corresponding naked template under the same assay conditions. Assembly took place at a weight ratio of histones to DNA equal to 0.8. Transcription was conducted as indicated under Experimental Procedures with the following variations: neither KCl nor spermidine were included, the concentration of Tris-HCl (pH 8.0) was 10 mM, and $MgCl_2$ was present at the indicated final concentrations. (B) Same results as in panel A, but the values for RNA synthesis shown in panel B are expressed relative to those obtained with the same template at 2 mM $MgCl_2$.

In the absence of spermidine and monovalent cations, the transcriptional efficiency of intact oligonucleosomes relative to the naked template increases with the concentration of Mg^{2+}. Figure 4A shows the RNA synthesis, relative to that of the corresponding free DNA, obtained with intact and trypsin-digested oligonucleosomes using pT207-18 and T7-207-18. The transcription efficiency of the intact oligonucleosomes at 4.5 mM $MgCl_2$ (approximately 3 mM free Mg^{2+}) is over 4 times larger than that found at 2 mM $MgCl_2$ (0.4 mM free Mg^{2+}) (Figure 4B). In contrast, the increase in efficiency obtained with the corresponding trypsin-digested templates is comparatively small (2 times and lower) (Figure 4B). Accordingly, the transcriptional stimulation due to elimination of histone tails decreases when the concentration of $MgCl_2$ in the assay mixture is increased from 2 to 4.5 mM. With the oligonucleosome assembled on T7-207-18, at concentrations of free Mg^{2+} (0.4 and 2 mM) equivalent to those present in the transcription assays with 2 and 3.6 mM $MgCl_2$, the values for the sedimentation coefficient (44S and 50S) indicate that the observed transcriptional stimulation by magnesium is accompanied by an increase in template compaction.

In an attempt to find assay conditions suitable to study transcriptional elongation, the transcription rate was decreased by lowering reaction time, temperature, and the concentrations of polymerase and nucleoside triphosphates (NTPs) in the assay mixture. Figure 5A shows the effect of temperature on the size of the transcription products obtained with naked pGEMEX-1; other assay conditions were those adopted for low reaction rate (see Experimental Procedures). The decrease in the amount and size of RNA with temperature reflects the fall in transcription rate. A temperature of 15 °C was chosen to evaluate the effects of histone octamers, intact and trypsin-digested, on chain elongation. The presence in the reaction mixture of different amounts of template does not affect the size of RNA obtained (Figure 5B). In addition, the total RNA synthesized was proportional to the amount of template. Therefore, complete inactivation of part of the templates, which can take place by blocking initiation or by aggregation, is not going to affect the size of the RNA synthesized on the remaining active templates. In agreement with the results obtained under conditions of high transcription rate (Figure 2B), the short RNA chains observed with both naked DNA and oligonucleosomal templates are not released from the transcription complexes but can be

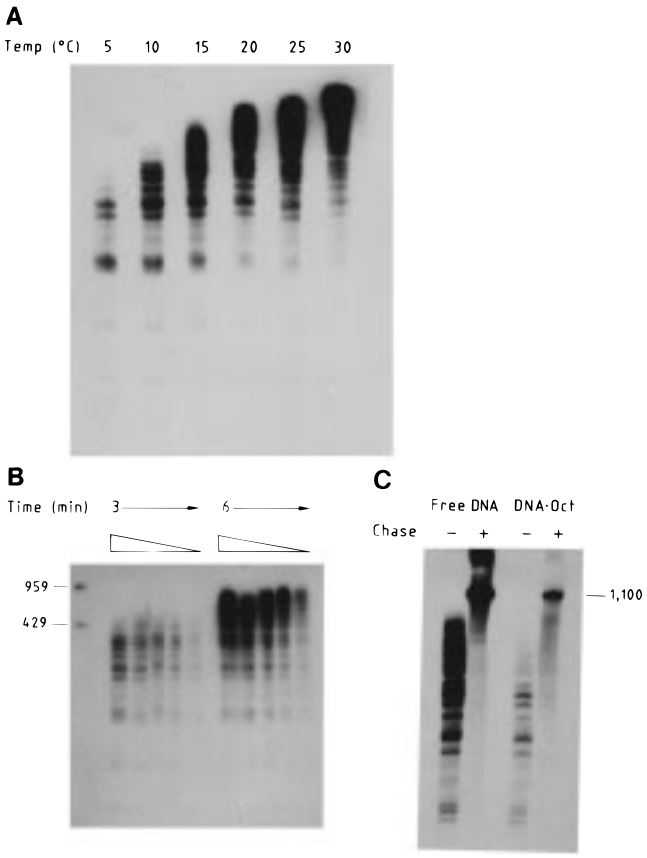

FIGURE 5: Transcription of pGEMEX-1 templates under low reaction rate conditions. (A) Effect of temperature on the size of the transcription products obtained with naked pGEMEX-1. Other reaction conditions were those used to have low transcription rate (Experimental Procedures). Incubation took place for 3 min. (B) Transcription products obtained with different amounts of naked pGEMEX-1. RNA synthesis took place for 3 and 6 min, under the low transcription rate conditions (Experimental Procedures), but with the following template concentrations: 1.9, 3.8, 5.6, 7.5, and 9.4 μg of DNA/mL. (C) Termination of small RNA chains transcribed from naked pGEMEX-1 and from the corresponding oligonucleosomes assembled at a weight ratio of histones to DNA equal to 0.8. Transcription took place under the low rate conditions (Experimental Procedures). After incubation for 5 min, transcription was ended in one of the two mixtures corresponding to each template (chase −), while unlabeled CTP, to a final concentration of 100 μM, was added to each of the second mixtures (chase +) and incubation was continued for another 7 min before stopping the reaction. The number on the right shows the size of the indicated band in nucleotides.

completed if transcription continues. Figure 5C shows how the observed short RNA chains disappear when transcription is maintained in the presence of an excess of unlabeled substrate, and all radiactivity becomes associated with high molecular weight RNA.

The transcription products obtained with intact and trypsin-digested oligonucleosome templates containing pGEMEX-1 as well as with the corresponding naked DNA are shown in Figure 6. At the two reaction times employed, the size of the RNA obtained with intact nucleosome templates is significantly smaller than that corresponding to the naked template, and the transcripts synthesized on templates lacking the histone tails have an intermediate size. The inclusion in the electrophoresis of appropriate markers of RNA size allowed determination of the number-average molecular weight of the synthesized RNA chains. From the number-average molecular weight of RNA and the amount of radioactive nucleotide incorporated into RNA, the relative number of RNA chains synthesized with each template can be calculated. Table 1 shows the values corresponding to 6 min reaction (Figure 6). If we assume that only the soluble templates can be potentially transcribed, from the solubility data a corrected set of values for RNA synthesis can be obtained as well as the corresponding relative numbers of transcripts. The corrected results show that only part of the overall transcription inhibition caused by the association with DNA of core histone octamers can be assigned to the observed decrease in elongation. The main contribution to inhibition seems to correspond to the blocking effect on

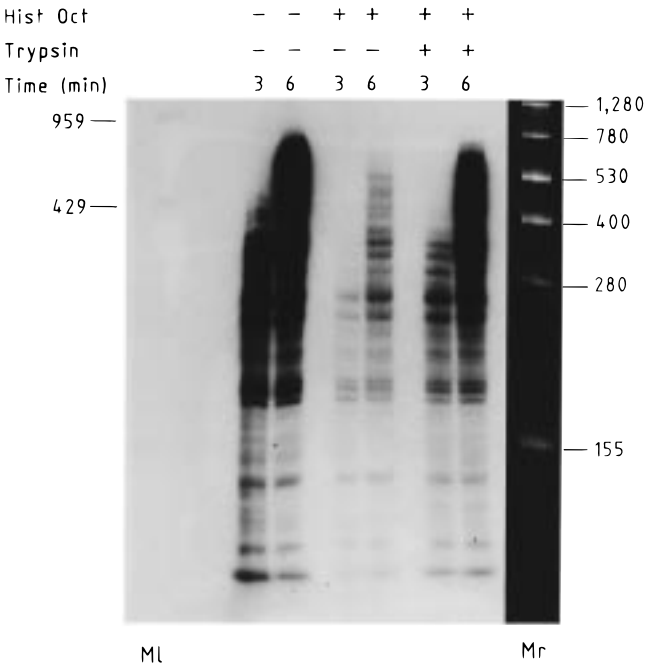

FIGURE 6: Transcription products obtained with intact and trypsin-digested oligonucleosomal templates assembled on pGEMEX-1. Assembly of oligonucleosome templates took place at a weight ratio of core histone octamers to DNA equal to 1. Transcription was allowed to proceed for 3 and 6 min under the low transcription rate conditions (Experimental Procedures). Size markers, radioactive (lane Ml) and nonradioactive (lane Mr), are included. The numbers on the right and left give the size of the markers in nucleotides.

Table 1: Number-Average Molecular Weight and Number of RNA Chains Synthesized on Intact and Trypsin-digested Oligonucleosomes Assembled on pGEMEX-1[a]

	number-average mol. weight			total RNA synthesis		no. of RNA
template	nucleotides	(%)	solubility (%)	determined (%)	corrected (%)	chains (%)
DNA	310	**100**	80	100	100	**100**
DNA·Oct	210	**68**	56	11	16	**24**
DNA·Oct, trypsin	263	**85**	88	54	49	**58**

[a] Data correspond to the experiment of Figure 6 (6 min transcription). The solubility of the different templates under conditions equivalent to those used in transcription are also included. The solubility data were used to correct total RNA synthesis for the different extents of aggregation of the templates and to obtain, from the corrected values for RNA synthesis, the number of RNA chains. In this way, the RNA synthesis and the number of chains corresponding to the same number of soluble templates were obtained. Values are expressed, except for the average length of RNA in nucleotides, as percentages of those corresponding to the naked template.

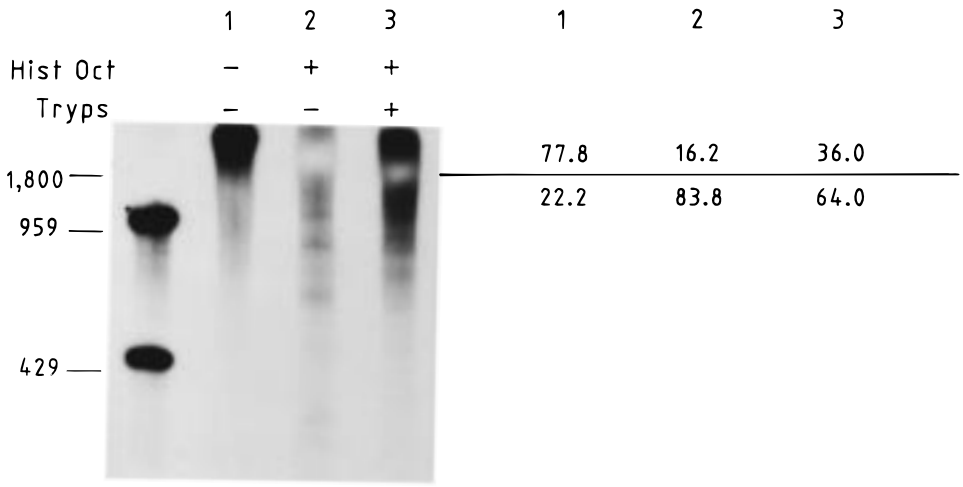

FIGURE 7: Transcription products obtained with intact and trypsin-digested oligonucleosomal templates assembled on T7-207-18. Assembly of oligonucleosome templates took place at a weight ratio of core histone octamers to DNA equal to 0.8. Transcription was allowed to proceed for 2 min in the presence of 67 mM KCl, 6 mM $MgCl_2$, and 2 mM spermidine (condition A, Figure 2). Other experimental details are those indicated under Experimental Procedures for high transcription rate, except that the concentration of ATP, GTP, and UTP was 0.1 mM and that of CTP was 25 μM. The percentage of the total radioactivity incorporated into RNA chains larger and lower than approximately 1800 nucleotides is indicated in the figure. The numbers on the left give the size of the indicated bands in nucleotides.

initiation, in agreement with previous results (*28*). The transcriptional stimulation that follows elimination of core histone tails appears to be mainly produced by relieving the inhibitory effect of core histones on initiation. A smaller but significant effect of trypsin digestion corresponds to easing elongation through nucleosomal structures.

The transcription conditions employed to study elongation of RNA chains with pGEMEX-1 templates proved unsuitable for pT207-18 and T7-207-18. However, changes in the initial assay conditions (high transcription rate under salt condition A) allowed detection of incomplete RNA chains with these templates as well as a rough estimation of the relative effects on elongation of intact and trypsin-digested oligonucleosomal templates. Figure 7 shows the transcription products obtained with free T7-207-18 and T7-207-18 associated with core histone octamers before and after elimination of the histone tails. Although with all three templates a significant part of the incorporated radioactivity appears as long RNA chains, with the two oligonucleosome templates shorter molecules are enriched relative to full-size chains. The proportion of radioactivity in short chains (smaller than 1800 nucleotides) relative to that found in long molecules (larger than 1800 nucleotides) is higher with the oligonucleosome template lacking the histone tails than with the corresponding intact template (Figure 7). Even with the trypsin-digested template, the relative presence of short chains is much larger than that corresponding to the naked template. These results, in agreement with those obtained with pGEMEX-1, indicate that the association of DNA with core histone octamers is accompanied by inhibition of transcript elongation, which is only partially relieved by elimination of the histone tails.

DISCUSSION

The mechanism of chromatin folding is primarily electrostatic in nature. Monovalent and divalent cations effect chromatin folding by reducing the residual DNA charge in different ways: monovalent cations by screening the residual charge and divalent cations by binding to DNA (*37*). In the absence of linker histones, regularly spaced nucleosomal arrays form partially folded structures at moderate NaCl concentrations (10−100 mM) (*11*, *36*), and a more folded conformation, equivalent to the 30-nm fiber, appears in the presence of 1−2 mM $MgCl_2$ (*38*). These two degrees of folding are prevented by the removal of the histone tails (*11*, *12*). Moreover, oligonucleosome self-association is induced by Mg^{2+} and other divalent cations, while monovalent cations reduce the extent of self-association at any given divalent cation concentration (*30*, *38*). As in the case of folding of the oligonucleosome chain, self-association requires the presence of the histone tails (*30*).

The folding and aggregation of nucleosomal arrays that take place in the presence of monovalent and divalent cations complicate the interpretation of transcription experiments. Hansen and Wolffe (*35*) reported that circular chromatin templates are compacted and even precipitated at the free Mg^{2+} concentrations used in transcription assays and that these phenomena are responsible for the inhibition they observed with RNA polymerase III. In our case, the possible aggregation induced by Mg^{2+} is critical, since trypsin digestion of oligonucleosomes increases their solubility in the presence of Mg^{2+} by preventing self-association (*30*). Therefore, the initially observed transcriptional stimulation that accompanies trypsin digestion might be caused by the presence of more molecules of soluble templates in the trypsin-digested than in the untreated preparation rather than by a removal of the transcription repression caused by the nucleosome structure.

Spermidine, routinely included in the in vitro transcription mixtures (*18*, *25*, *28*), and other polyamines are known to induce aggregation of DNA and oligonucleosomes (*41*, *42*). Our results show that spermidine is more effective than Mg^{2+} in promoting aggregation of intact oligonucleosomes (Figure 3A,C) and that both K^+ and Mg^{2+} reduce this effect, probably by displacing spermidine from DNA. Because of this effect of K^+ and Mg^{2+}, under the usual transcription conditions (67 mM KCl and 6 mM $MgCl_2$), spermidine does not have

a significant effect on aggregation. As in the case of the self-association induced by Mg^{2+} (*30*), elimination of the tails of core histones prevents aggregation of oligonucleosomes by spermidine. The similarities with the action of Mg^{2+} suggest that both spermidine and magnesium ions induce oligonucleosome self-association in a similar way. Schwarz et al. (*30*) proposed that the mechanism governing the in vitro self-association of oligonucleosomes by Mg^{2+} also participates in the stabilization of higher order chromosomal fibers in vivo. Spermidine and other polyamines, which are common and relatively abundant cellular compounds (*41*), might play a role in chromatin compaction in vivo alongside of Mg^{2+}.

Elimination of the histone terminal domains from oligonucleosome templates has been shown to facilitate RNA synthesis under different salt conditions and in the presence and absence of spermidine (Figures 2A and 4). Differences in self-association between intact and trypsin-digested templates are not the only cause of the observed stimulation, since it persists after the values of RNA synthesis are corrected to take into consideration only the amount of soluble template. However, folding of oligonucleosomes to give condensed soluble structures might be responsible for the different transcription efficiencies obtained with intact and trypsin-digested templates, since the histone tails are required to fold oligonucleosomes to the 30-nm fiber and the condensed intermediate (*11, 12, 40*). In the absence of spermidine and KCl, maximal stimulation by the absence of histone tails is observed at low concentration of free Mg^{2+} (0.4 mM over the concentration of substrate nucleotides) when compaction of the intact oligonucleosome (44S) is lower than that observed at 2 mM free magnesium (50S), when the transcriptional efficiency of the intact oligonucleosome is largest (Figure 4). Since the apparent stimulation by absence of histone tails is largest at the lowest magnesium concentration employed, when the difference in folding between intact and trypsin-digested templates is the smallest, it appears that stimulation is caused at the nucleosome level rather than by a decrease in folding. Moreover, an increase in compaction of the intact oligonucleosome is not accompanied by transcriptional repression (Figure 4). Using a saturated 208-12 oligonucleosome, Fletcher and Hansen (*12*) conclude that histone tails are released from their nucleosomal location in 2 mM $MgCl_2$ concomitant with the formation of folded oligonucleosome structures. The tails presumably mediate folding by interacting with oligonucleosomal constituents other than nucleosomal DNA. The release of the histone tails from a nucleosomal location might facilitate transcription by overcoming the presumably negative effect of folding, which would explain the found magnesium-dependent increase in transcription.

It is generally accepted that transcription can take place through nucleosomal structures and that elongation is retarded by an increase in the time spent by RNA polymerase at natural transcription pauses (*13*). Premature termination and release of incomplete RNA chains might also take place in the presence of nucleosomal structures. Our results with pGEMEX-1 show that the decrease in chain length observed in the presence of histone octamers, under the employed low transcription rate conditions, is not caused by an abortive release of incomplete chains since the labeled short RNA chains disappear when transcription continues in the presence of an excess of unlabeled substrate, and most of the corresponding radioactivity is present as long chains (Figure 5C). With all used templates (Figure 2B), after extensive transcription at high rate, most of the incorporated radioactivity appears as long RNA chains, suggesting that if abortive release takes place it is a relatively rare event.

The decrease in average chain length of synthesized RNA that accompanied the association of histone octamers with DNA is different with pGEMEX-1 and pT207-18 for templates assembled at a weight ratio of histones to DNA equal to 1 and transcription conducted under similar conditions (*18, 28*). The average chain length of the RNA obtained with the oligonucleosome template relative to that synthesized with the corresponding naked DNA is 30% for pT207-18 (*18*) and 80% for pGEMEX-1 (*28*). These results indicate that the inhibition of chain elongation is much larger with oligonucleosome templates assembled on pT207-18 than with those obtained with pGEMEX-1. In addition to the possible contribution of folding and compaction to inhibition, the differences between the results obtained with the two oligonucleosomal templates might be due to the different sizes of the corresponding transcription products. pGEMEX-1 has a coding region of 1100 bp, located between the promoter and a terminator. pT207-18 has no terminator, and with the free template a maximum average transcript length of 4600 nucleotides is obtained, transcription exceeding the 3700 bp of the nucleosome positioning region (*18*). Therefore, RNA polymerase has to transcribe through a larger number of nucleosomes with pT207-18 (18−23 nucleosomes) than with pGEMEX-1 (5−6 nucleosomes), which might explain the larger inhibition of elongation observed with pT207-18. The pause of the transcription machinery at each nucleosome would hinder transcription by the following polymerases, and inhibition would increase with the size of the transcribed oligonucleosome.

Analysis of the electrophoresis of transcription products obtained with pGEMEX-1, under conditions of low reaction rate (Table 1), shows a decrease in the number of transcripts caused by the association with DNA of core histone octamers, which indicates a parallel decrease in the number of active templates, presumably blocked at the initiation step. This inhibition is substantially relieved in the absence of histone tails. Although the effect of histone octamers on chain elongation is clearly detected, it makes a relatively small contribution to the overall inhibition. Therefore, under the employed conditions, the stimulatory effect accompanying elimination of histone tails in oligonucleosomes assembled on pGEMEX-1 is mainly accomplished by deblocking initiation, although a significant increase in elongation rate is also found.

With T7-207-18, analysis of the transcripts, obtained at 37 °C but under conditions that lowers the transcription rate, shows that histone octamers, intact or lacking their terminal tails, cause a large inhibition of elongation (Figure 7). This inhibitory effect is larger with intact histones than with those trypsin digested, indicating that, as with pGEMEX-1, the histone terminal domains contribute to the repression of elongation. However, because of the larger size of the transcribed DNA in pT207-18 as compared to pGEMEX-1, with T7-207-18 the effect of histone octamers on elongation has a larger share in the overall inhibition, in agreement with previous data (*18, 28*).

Our results show that the terminal domains of core histones play a significant role in the repression of in vitro transcription by nucleosomal structures. At least a significant part of the observed effect takes place at the nucleosome level, the interaction of the histone terminal domains with DNA probably hampering the action of the transcription machinery. The repressive effect of the histone terminal domains on transcription could be modulated by changing their interaction properties. Accordingly, acetylation of histone amino-terminal tails has already been found to facilitate transcription in vitro (*23*, *24*, *27*, *39*).

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