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Inhibition of 5S RNA transcription in vitro by nucleosome cores with low or high levels of histone acetylation

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Nucleosomes exert strong inhibitory effects on gene transcription in vitro and in vivo. Since most DNA is packaged in nucleosomes, there must exist mechanisms to alleviate this inhibition during gene activation. Nucleosomes could be destabilized by histone acetylation which is strongly correlated with gene expression. We have compared the effects of nucleosomes cores with low or high levels of histone acetylation on 5S RNA transcription with *Xenopus* nuclear extracts in vitro. Little or no difference was observed over a range of 1 to 15 nucleosome cores per plasmid template. This result suggests that nucleosomal DNA is not more accessible to transcription factors and to the transcription machinery in acetylated nucleosomes.

Histone acetylation; 5S RNA; In vitro transcription; Gene activation; Nucleosome

1. INTRODUCTION

Eukaryotic DNA is packaged into chromatin, the basic repeated unit of which is the nucleosome. Long-standing in vitro evidence and recent in vivo data show that DNA in nucleosomes is not accessible to DNA-binding proteins such as transcription factors, and that nucleosomes inhibit transcription initation (see [1,2] for recent reviews). How then do transcription factors and RNA polymerases gain access to their cognate sites in chromatin in order to activate transcription?

There is evidence that certain key nucleosomes are displaced when genes are activated. For example, induction of the PHO5 gene in yeast causes the displacement of two nucleosomes which are precisely positioned on either side of an essential promoter element [3,4]. In a second example, binding of the glucocorticoid receptor to the mouse mammary tumor virus promoter leads to the displacement of a nucleosome and to the establishment of a DNase I hypersensitive site when transcription is activated [5,6].

Displacement of nucleosomes could be facilitated by reversible histone modifications which destabilize the nucleosome. Acetylation is the reversible histone modification which is strictly correlated with gene expression [7–10]. During the cell cycle of *Physarum polycephalum* incorporation of labeled acetate into histone H4 increases during periods of gene activity [11]. Active chromatin released preferentially by nuclease digestion is enriched in acetylated histones [12–15]. Active chromatin selectively retained by mercurial agarose is likewise enriched in acetylated histones H3 and H4 [16] and this retention is tightly correlated with gene activity: histone genes are retained during S-phase and not G2 phase [9], and *c-myc* and *c-fos* genes are retained only and precisely during their periods of expression [17].

The amino-terminal domains of the four core histones contain all the sites of reversible acetylation: one site for histone H2A, four sites for histones H2B and H4, and five sites for histone H3 [7,18]. Acetylation of these sites causes an important reduction in the net positive charge of histone octamers and affects the thermal stability of the DNA in nucleosomes [19]. These effects may weaken the association of histones with nucleosomal DNA, and could facilitate nucleosome displacement by transcription factors.

In this study, we have addressed the following question. Does hyperacetylation of the histone octamer in 5S RNA genes assembled with nucleosome cores increase their transcriptional efficiency compared with nucleosome cores with low levels of acetylation? The *Xenopus laevis* 5S RNA gene was chosen for this study because: (1) a nucleosome is positioned precisely on the promoter region, with its center at or near the start site of transcription, following assembly of nucleosome cores onto 5S RNA genes in vitro [20–23]; (2) very efficient in vitro transcription can be performed with nuclear extracts that do not themselves assemble chromatin on added template DNA [24]; (3) the very

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small size of the gene, which accommodates only one nucleosome, permits analysis of the effects of nucleosomes on transcription initiation, without additional effects on transcription elongation; (4) acetylated and non-acetylated nucleosomes assembled onto a related 5S RNA gene have been extensively characterized biochemically [18].

2. MATERIALS AND METHODS

2.1. *Isolation of nucleosomal core particles with low or high levels of histone acetylation*

Nucleosomal core particles were isolated from HeLa cells according to [25]. Briefly, nuclei isolated from cells treated with or without 7 mM sodium butyrate were digested with micrococcal nuclease and lysed with EDTA. This released typically 40% of the total chromatin. The soluble chromatin was subsequently depleted of histone H1 by chromatography on hydroxylapatite, and then redigested with micrococcal nuclease to produce core particles which were isolated by ultracentrifugation through a sucrose gradient. Finally, core particles with different levels of histone acetylation were separated by chromatography on hydroxylapatite. The levels of histone acetylation were determined by electrophoresis of 30 μg core particles on 12% polyacrylamide Triton-acid-urea gels followed by scanning using a Molecular Dynamics Model 300A computing densitometer [26].

2.2. *Nucleosome core reconstitution*

Nucleosome cores were reconstituted onto supercoiled plasmid pXp14 [27] using a salt dilution method. In a typical experiment, donor core particles were mixed with 4.2 μg plasmid pXp14 in an initial volume of 10 μl in buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 0.1 mM phenylmethylsulfonylfluoride) containing 2 M NaCl. Samples were incubated at room temperature for 20 min and were then diluted with buffer at successive intervals of 30 min to 1.12 M, 0.8 M, and 0.6 M NaCl. Samples were further diluted to 0.15 M NaCl by addition of 10 mM Tris-HCl pH 7.6 and were used immediately for most in vitro transcription experiments, or kept on ice for a maximum of a few days.

2.3. *Extent of nucleosome core assembly*

The numbers of nucleosome cores assembled onto plasmid pXp14 were deduced from the numbers of constrained negative supercoils in minichromosomes relaxed with topoisomerase I by electrophoresis in agarose gels containing chloroquine diphosphate [28]. Minichromosomes reconstituted from 3 μg of plasmid DNA were incubated for 1 h at 37°C with chicken erythrocyte topoisomerase I (prepared as in [25], and kindly provided by Dr P. Yau) at 15 U/μg input plasmid in 10 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$. SDS and EDTA were added to 0.2% and 15 mM, respectively, followed by extraction with phenol-chloroform and precipitated with ethanol. The samples were separated into thirds and electrophoresed in 1% agarose gels containing 0.75, 1.5, or 6 μg/ml chloroquine diphosphate for 16 h at 60 V. After electrophoresis, the gels were soaked in several changes of water, stained with ethidium bromide, and extensively destained in water. The center of the distribution of topoisomers in each sample was determined from scanned photographic negatives of the gels by band counting [29] and using the algorithm of Kolb and Buc [30].

2.4. *Transcription reactions*

5S RNA gene transcription was performed with extracts prepared from manually isolated germinal vesicles obtained from stage 4 and 5 oocytes of mature *Xenopus laevis* [24,31]. Germinal vesicles were stored at −70°C in J buffer (70 mM NH_4Cl, 7 mM $MgCl_2$, 0.1 mM EDTA, 2.5 mM dithiothreitol, 10% (v/v) glycerol, 10 mM HEPES, pH 7.4). The germinal vesicle extracts were prepared immediately before use by disrupting freshly thawed germinal vesicles by pipetting 5–10 times using the yellow tip of a micropipettor. The lysate was cleared of nuclear debris by centrifuging at 12 000 × g for 30 s at 4°C.

Prior to transcription, plasmid DNA or minichromosomes were first treated with topoisomerase I to relieve unconstrained negative supercoils. For each reaction, 25 ng plasmid pXp14, reconstituted or not with nucleosomes, were incubated with 0.2 U topoisomerase I for 15 min at 25°C in 2.5 μl J buffer containing 3 mM each ATP, CTP, UTP, 0.1 mM GTP and 20 μM [α-^{32}P]GTP (3 μCi/reaction). Transcription was initiated by adding 3.2 μl germinal vesicle extract and was carried out for 30 or 60 min at 25°C.

Transcription reactions were stopped by the addition of 50 μl 0.1% SDS, 5 mM EDTA, 0.25 M sodium acetate, pH 5.7 containing 20 μg/ml carrier tRNA. After extraction with phenol and ethanol precipitation, the pellets were solubilized in 90% deionized formamide containing xylene cyanol and Bromophenol blue and heated at 90°C for 5 min. Samples were analysed by electrophoresis in 8% polyacrylamide gels containing 8.3 M urea. The gels were dried and the amount of radioactivity in the 5S RNA bands was quantitated with an AMBIS Radioanalytical Imaging System, AMBIS Systems Inc., San Diego, CA.

3. RESULTS

3.1. *Minichromosomes with low or high levels of histone acetylation*

In order to study the effect of histone acetylation on 5S RNA transcription, plasmid pXp14, which contains one copy of a *Xenopus borealis* somatic type 5S RNA gene, was assembled into chromatin with histones of low or high acetylation levels. Nucleosome core particles were isolated from butyrate-treated HeLa cells and separated according to their level of acetylation by hydroxylapatite chromatography. Core particles with the highest and lowest levels of acetylation were used as donors of histone octamers in the assembly reaction. Electrophoresis in Triton-acid-urea gels was used to resolve the differently acetylated histones (Fig. 1). Densitometric analysis of these Coomassie blue-stained gels gave average acetylation levels of 2.6 and 0.75 per

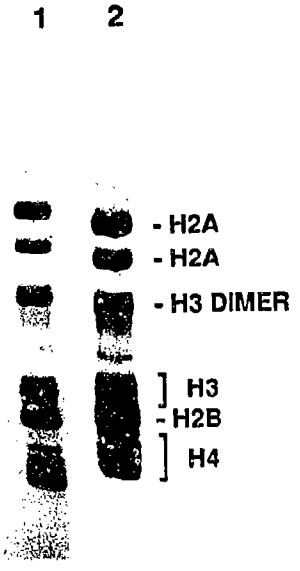

Fig. 1. Triton-acid-urea gel electrophoresis of histones from core particles with low (lane 1) or high (lane 2) levels of histone acetylation.

histone H4 molecule in core particles with high and low levels of acetylation, respectively.

Minichromosomes containing different numbers of nucleosome cores were obtained using a salt dilution method and different weight ratios of donor core particles to acceptor pXp14 DNA. The extent of nucleosome reconstitution was determined from the numbers of constrained negative supercoils, after relaxation of samples with topoisomerase I, deproteinization, and electrophoresis in agarose gels containing chloroquine disphosphate. The centers of the topoisomer distributions were determined by densitometric analysis of photographic negatives of these gels. Nucleosome cores with low and high acetylation levels constrain 1.04 ± 0.08 and 0.82 ± 0.05 negative supercoils, respectively [25,32]. Minichromosomes containing between one and 12 nucleosome cores were obtained. It has been observed previously that highly acetylated nucleosome cores are assembled more efficiently on DNA than those with lower levels of acetylation [25,33]. This was also observed in our study and the input ratios of donor core histones to pXp14 DNA was adjusted in order to produce minichromosomes with comparable numbers of nucleosomes.

3.2. *In vitro transcription*

Reconstituted minichromosomes were treated with topoisomerase I in order to generate fully relaxed templates for transcription. The relaxed minichromosomes were then incubated with an oocyte nuclear extract which can transcribe 5S RNA genes very efficiently.

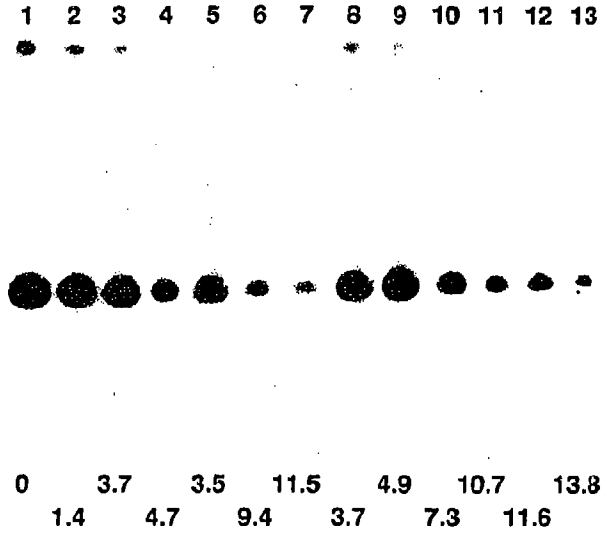

Fig. 2. Analysis of 5S RNA transcripts from plasmid pXp14 reconstituted with different numbers of nucleosomes. Transcripts were analyzed by electrophoresis in 8% polyacrylamide gels containing 8.3 M urea, followed by autoradiography. Lane 1, control naked plasmid; lanes 2–7, nucleosomes with low levels of acetylation; lanes 8–13, nucleosomes with high levels of acetylation. The numbers of nucleosomes determined from the number of constrained negative supercoils are indicated at the bottom.

Fig. 2 shows an autoradiogram of radiolabeled 5S RNA transcribed in vitro by minichromosomes containing different numbers of nucleosome cores. The presence of nucleosomes on the templates had a strong inhibitory effect on 5S RNA transcription. The inhibition increased as the numbers of nucleosome cores in the minichromosomes increased. It is clear that highly acetylated nucleosome cores also inhibited transcription.

The amounts of radioactive 5S RNA were quantitated by scanning directly the dried gels with an AMBIS β counter. Fig. 3 shows a plot of the inhibition of transcription as a function of nucleosome core number for minichromosomes containing low or high levels of acetylated histones. For equal numbers of nucleosome cores, there was little or no difference in the extent of inhibition by histone octamers with low or high levels of acetylation. 50% inhibition was obtained at 4–5 nucleosomes per tempalte. This experiment was performed three times with similar results.

4. DISCUSSION

In this study, we have tested the hypothesis of whether transcription is more efficient when templates contain nucleosome cores with high levels of histone acetylation rather than low levels of acetylation. We have shown that for 5S RNA transcription performed in vitro with a *Xenopus laevis* oocyte nuclear extract, nucleosome cores with high or low levels of histone acetylation are strongly and comparably inhibitory to transcription. Mathis et al. [34] observed that histone acetylation had no apparent effect on transcription when isolated HeLa hyperacetylated chromatin was transcribed in a non promoter-specific fashion by *E. coli* RNA polymerase or mammalian RNA polymerase

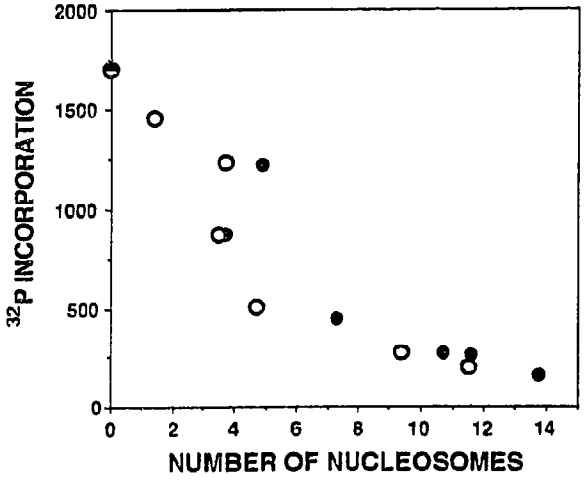

Fig. 3. Quantitation of 5S RNA transcription as a function of the numbers of nucleosomes assembled onto the plasmid templates.

I or II. Our study, which examines RNA polymerase III transcription initiated at a eukaryotic promoter, confirms and extends their observation.

Our result is likely to represent inhibition at the level of transcription initiation rather than elongation because this small gene can accommodate only one nucleosome which appears to be absent from chromatin containing active 5S RNA genes. Furthermore the inhibition is probably due to lack of binding of transcription factors; the transcription factor TFIIIA has been shown to be unable to bind a 5S RNA gene occupied by a positioned nucleosome in chromatin reconstituted with a S-150 extract [23]. This is in agreement with most previous studies [31,35,36], but not all [21]. In this respect, it is interesting to note than an acetylated nucleosome core is positioned with the same periodicity as a non-acetylated nucleosome core on the 5S RNA gene of *Lytechinus variegatus*, as determined by DNase I footprinting of in vitro-reconstituted nucleosome cores [18].

However, although our results show that the efficiency of 5S RNA transcription by RNA polymerase III is not enhanced by acetylation of the histone octamer in nucleosome cores, they do not rule out participation of histone acetylation in the process of gene activation. For example, the nuclear extracts used in this study could lack factors present in vivo which are necessary for an effect of acetylation on transcription. In addition, histone acetylation could participate in gene activation through some other mechanism. A possible mechanism is that acetylation weakens the binding of histone H1, destabilizing the higher order structures of chromatin, e.g. the 30 nm supercoil of nucleosomes, thus facilitating access to transcription factors and to the transcription machinery. Histone acetylation could also affect the positioning or spacing of key regulatory nucleosomes at a distance from the transcription start site of a gene, as suggested by Turner et al., [37], who found that acetylated histone H4 localizes to specific regions adjacent to the boundaries of decondensed, transcriptionally active chromatin in *Chironomus*. These possibilities could be investigated by examining the effects of histone acetylation on gene activation in chromatin containing higher order structures assembled in vitro with histone H1 and other factors.

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