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Characterization of the Repressed 5S DNA Minichromosomes Assembled In Vitro with a High-Speed Supernatant of *Xenopus laevis* Oocytes

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We describe an in vitro system, based on the *Xenopus laevis* oocyte supernatant of Glikin et al. (G. Glikin, I. Ruberti, and A. Worcel, Cell 37:33–41, 1984), that packages DNA into minichromosomes with regularly spaced nucleosomes containing histones H3, H4, H2A, and H2B but no histone H1. The same supernatant also assembles the 5S RNA transcription complex; however, under the conditions that favor chromatin assembly, transcription is inhibited and a phased nucleosome forms over the 5S RNA gene. The minichromosomes that are fully loaded with nucleosomes remain refractory to transcriptional activation by 5S RNA transcription factors. Our data suggest that this repression is caused by a nucleosome covering the 5S RNA gene and that histone H1 is not required for regular nucleosome spacing or for gene repression in this system.

The bulk of the DNA in a differentiated cell of higher eucaryotes, unlike the DNA of procaryotes, is packaged by histones and other proteins into compact nucleosome and supranucleosome structures that are not readily accessible for genetic readout (see reference 65 for a review). These nucleoprotein structures are assembled during chromatin replication via pathways that are still poorly understood. Once assembled, the structures are usually stable throughout the life span of the cell, and, at least in a few documented cases, they can apparently also propagate through cell division. One example of such an inheritable chromatin structure is the compacted and inactive X chromosome in female mammals, faithfully inherited by all the descendents of the blastomere in which the initial inactivation event occurred (39).

A purely descriptive analysis of chromatin may never reveal the mechanism responsible for generating those remarkable structures, and, furthermore, lack of knowledge of the mechanism will continue to hamper efforts to understand eucaryotic gene expression and its regulation. It was previously shown that DNA can be assembled into chromatin by *Xenopus laevis* oocytes in vivo and in vitro (35, 36, 40, 72). With the hope of developing a mechanistic approach to chromatin studies, investigators in this laboratory began an analysis of the process of nucleoprotein assembly as it occurs on DNA introduced into *Xenopus* oocytes (15, 17, 18). A series of experiments performed first in vivo (16, 51, 52) and then in vitro in a cell extract (oocyte S:150 [20, 50]), suggested that assembly occurs via a gradual and complex maturation process that can produce two types of chromatin, one active (dynamic) and the other one inactive (static).

Subsequent in vitro work indicated that the 5S-specific transcription factor IIIA (TFIIIA) could induce the formation of dynamic chromatin in oocyte S-150 under particular experimental conditions (27–30). Those experiments are complicated, and we and others (66) have not been able to reproduce them. Previous work was performed on trace amounts of chromatin assembled on [^{32}P]DNA circles labeled to high specific activity, which is not practical. We have attempted to simplify these studies by developing in the present work a more reproducible system for assembling chromatin in sufficiently large amounts to allow subsequent biochemical analysis.

Native chromatin is a periodic structure made up of regularly spaced subunits, as was first revealed when nuclei were digested with endonucleases (22). Since then, many attempts have been made to reconstitute this structure in vitro. One approach has been to incubate DNA with histones in the presence of counterions, such as NaCl (19), nucleoplasmin (34), polyglutamic acid (59), and RNA (43), which presumably lessen electrostatic interactions and facilitate a gradual nucleoprotein assembly. The nucleosomal subunits of chromatin are reconstituted under these conditions, but the native periodic structure usually is not formed [but see references 57 and 58 for the two special cases of sea urchin 5S DNA and poly(dA-dT) · poly(dA-dT) DNA tandemly repeated].

The other approach has been to use cell extracts prepared from *Xenopus* eggs (36) or *Xenopus* oocytes (20), which contain a relatively large pool of histones, to assemble chromatin by appropriate incubations with exogenously added DNA. Chromatin with nucleosomes regularly spaced at about 190-base-pair (bp) intervals is formed under these conditions, but the extracts are not simple to use and the DNA is not always fully loaded with nucleosomes after such incubations. Moreover, the reaction works best at high surface-to-volume ratios and in small incubation volumes of 5 to 10 μl (20, 36), requiring labeled DNA probes for the analysis of the small amounts of chromatin formed (see, e.g., references 20, 31, 33, 35, 36, 50, and 70).

We have succeeded in scaling up the chromatin assembly reaction (A. Shimamura, B. Jessee, and A. Worcel, Methods Enzymol., in press), thus greatly facilitating the analysis by eliminating the requirement for labeled DNA circles. Using these new protocols, we have assembled in oocyte S-150 5S DNA minichromosomes that are either transcribable or nontranscribable. We present simultaneous studies on DNA topology, protein composition, and nucleosome spacing and phasing on the in vitro-assembled minichromosomes. Such a detailed and integrated approach is crucial if we are to understand the mechanisms by which eucaryotic genes are regulated.

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MATERIALS AND METHODS

Preparation of oocyte S-150. Oocyte S-150 extracts were prepared from adult *X. laevis* female frogs (Nasco) essentially as described by Glikin et al. (20) and Shimamura et al. (in press), with slight modifications. Ovaries were washed in OR-2 medium (20) and digested for 3 h with 0.15% collagenase type II (Sigma Chemical Co.) in OR-2 medium at room temperature. The dispersed oocytes were washed 10 times in OR-2 medium and then 3 times at 4°C in low-ionic-strength extraction buffer [20 mM HEPES, (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.5), 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA), 10% glycerol, 10 mM β-glycerophosphate, 0.5 mM dithiothreitol]. The Beckman SW 50.1 centrifuge tubes were completely filled with oocytes and centrifuged at 40,000 rpm (150,000 × *g*) for 2 h at 4°C. The extended centrifugation pelleted the ribosomal subunits, which otherwise comigrate with minichromosomes in the preparative sucrose gradients. The clear supernatant (ca. 1 ml per 5-ml centrifuge tube) was removed with a syringe through the side of the centrifuge tube. Phenylmethylsulfonyl fluoride (final concentration, 20 μg/ml), leupeptin (final concentration, 2 μg/ml), and pepstatin (final concentration, 2 μg/ml) were added and gently mixed with the supernatant. Portions (200 to 400 μl) were stored at −70°C.

Plasmid DNA. Plasmid pXbsF201 carries the 240-bp *Xenopus borealis* somatic 5S RNA gene inserted in the *Hin*dIII-*Bam*HI site of pUC9 (47). The somatic 5S maxigene, pXbs115/105 in pUC (5), was the gift of D. Bogenhagen. The bacterial strain DH5α, which is recombination deficient, was used in preparing the plasmids to obtain predominantly the monomer form of the plasmid.

Chromatin assembly reaction. The chromatin assembly reactions were performed essentially as described by Shimamura et al. (in press), except that fresh additional phenylmethylsulfonyl fluoride (final concentration, 100 μg/ml), leupeptin (final concentration, 2 μg/ml), and pepstatin (final concentration, 2 μg/ml) were added. The DNA and all other reagents were dissolved in the extraction buffer. ATP (final concentration, 3 mM) $MgCl_2$ (final concentration, 5 mM) disodium creatine phosphate (final Na^+ concentration, 80 mM; final PO_4^{3-} concentration, 40 mM; Boehringer Mannheim Biochemicals), creatine phosphokinase (final concentration, 1 ng/μl; Boehringer Mannheim), and oocyte S-150 (to a final reaction volume of 60%) were added, and the mixture was incubated at 37°C. All extracts were tested for their capacity to assemble chromatin by DNA supercoiling and micrococcal nuclease (MNase) digestion analyses. When oocyte S-150 is prepared as described here, 1 μl of S-150 should contain about 2.5 μg of protein and should assemble up to 5 ng of DNA into nucleosomal chromatin. We have observed that somewhat higher DNA inputs can still be supercoiled in these extracts, but a regular and clear nucleosomal periodicity is not obtained under those conditions. Thus, the amount of DNA used should not exceed the capacity of the S-150 to produce a regular MNase ladder.

For the supercoiling assays, reactions were stopped by the addition of sodium dodecyl sulfate (SDS; final concentration, 0.2%) and EDTA (final concentration, 16 mM). The DNA was deproteinized with 1 mg of proteinase K per ml, at 37°C for at least 2 h. The DNA can be loaded directly onto 1% agarose gels in Tris-glycine buffer or concentrated by precipitation with ethanol prior to electrophoresis. It was visualized in the gel by staining with ethidium bromide.

Micrococcal nuclease digestions. Digestion of the minichromosomes with MNase was performed as described by Shimamura et al. (in press). Following chromatin assembly, the reaction mixtures were cooled to room temperature, and $CaCl_2$ (final concentration, 3 mM) and MNase (Boehringer Mannheim) were added. Aliquots were removed at various times, as indicated in the figure legends, and the digestion was stopped with 1/4 volume of a 2.5% sarcosyl–100 mM EDTA solution. A 1-μl portion of a 10-mg/ml RNase A solution (DNase-free) was added, and the tubes were incubated at 37°C for 10 min. SDS (final concentration, 0.2%) and proteinase K (final concentration, 1 mg/ml) were added, and the tubes were incubated for at least 4 h at 37°C. The DNA was precipitated with ammonium acetate (final concentration, 3 M) and 2 volumes of ethanol at −70°C for 30 min. The DNA was pelleted for 15 min in a microcentrifuge, and the pellets were washed twice with 80% ethanol, dried under reduced pressure, and dissolved in 10 mM Tris–1 mM EDTA. Sample dye (5× contains 50% glycerol, 0.1% orange G, and 5 mM EDTA) was added, and the samples were electrophoresed on 1.5% agarose gels in Tris-glycine buffer until the dye had run two-thirds the length of the gel (12 to 13 cm). The gels were stained with ethidium bromide and destained in distilled water. They were illuminated with short-wave UV light and photographed with Polaroid Land Film (type 55). Orange G dye was used because it was not visible in the photographs. Optimal resolution of the oligonucleosome-size DNA fragments was obtained with very small, narrow wells, such as those produced by a 30-well comb (170-4344; Bio-Rad Laboratories).

The nucleosome repeat length was determined on the 2- and 4-min digestion samples (see Fig. 2) as follows. The size of the larger visible oligonucleosomes was determined by using the molecular weight markers; the length in base pairs of each oligonucleosome was then divided by the number of nucleosomes it contained. This approach minimizes the effect of exonucleolytic trimming by MNase.

Two-dimensional gel electrophoresis of DNA topoisomers. Minichromosomes assembled for various times were deproteinized in SDS (final concentration, 0.2%)–EDTA (final concentration, 16 mM)–proteinase K (final concentration, 1 mg/ml) at 37°C for 30 min. The DNA was extracted twice with phenol-chloroform and twice with chloroform and was precipitated with 0.3 M sodium acetate and 2 volumes of ethanol. The DNA was pelleted, washed twice with 80% ethanol, and suspended in 100 μl of 10 mM Tris (pH 7.5)–1 mM EDTA (TE). Ammonium acetate (final concentration, 3 M) and 2 volumes of ethanol were added, and the DNA was pelleted, washed twice with 80% ethanol, dried under reduced pressure, and suspended in TE. Two-dimensional gel electrophoresis was performed as described by Peck and Wang (46). The DNA was electrophoresed in a 1% agarose gel (20 cm by 20 cm) in TBE (89 mM Tris-borate, 2 mM EDTA) at 60 V for 16 h. The gel was then soaked for 6 to 8 h in 2 liters of TBE containing 4 μM chloroquine in the dark, turned through 90°, and electrophoresed for 16 h at 60 V in the dark with buffer recircularization. The temperature of the buffer during the electrophoresis was 30°C. The gels were washed with distilled water and stained with ethidium bromide. The negatives were reverse-printed, to increase resolution (46).

The standard topoisomer ladder was prepared by relaxing the supercoiled 5S DNA plasmid with topoisomerase I in the presence of various concentrations of ethidium bromide by the method of Keller (24). The reference relaxed DNA (ΔL [linking number] = 0) was prepared by relaxing the supercoiled 5S DNA plasmid with topoisomerase I at 37°C in

extraction buffer plus 3 mM ATP, 5 mM $MgCl_2$, 40 mM creatine phosphate, 1 ng of creatine phosphokinase per μl, and proteolytic inhibitors. Duplicate samples were incubated for 1 h with 10 and 20 U of topoisomerase I to ensure that the DNA relaxation was complete. No further topoisomer shift was observed between these two samples.

Isolation of the minichromosomes. Linear 15 to 30% sucrose gradients in 100 mM NaCl–5 mM HEPES (pH 7.5)–0.2 mM EGTA were made in Beckman SW41 centrifuge tubes to a final volume of 11 ml. A 1-ml sample of the chromatin assembly reaction mixture or a control reaction mixture (same conditions as the chromatin assembly reaction but in the absence of DNA) was layered on top of the gradients and centrifuged at 22,000 rpm for 16 h at 4°C. Fractions (0.5 ml) were collected, and the position of the minichromosomes was determined by electrophoresis on 1.5% agarose gels and staining with ethidium bromide (see Fig. 4).

Electron microscopy. A sample of the fraction containing the minichromosomes was diluted fivefold with the gradient buffer, and the unfixed sample was applied to a glowed carbon-coated grid (11), which was stained with a dilute solution of uranyl acetate (0.01% in 90% ethanol). The grid was subsequently rotary-shadowed with platinum at an angle of 8°. The micrographs were taken at a magnification of ×50,000 with a Zeiss EM109 microscope.

Protein analysis. Fractions from the sucrose gradients containing the minichromosomes were pooled. Corresponding fractions from the control gradients (no DNA) were also pooled. These fractions were layered over a 1-ml cushion of 30% sucrose in gradient buffer and pelleted in a Beckman SW60 rotor at 30,000 rpm for 12 to 16 h. The supernatant was removed, and the pellet was suspended in the appropriate loading buffer and stored at −70°C. For SDS-polyacrylamide gels, electrophoresis in 15% slab gels was carried out by the method of Laemmli (32).

For two-dimensional gel electrophoresis, the sample was first electrophoresed in 15% polyacrylamide–Triton–acid–urea tube gels, essentially as described by Alfageme et al. (2). To free the histones from the DNA, we added an equal volume of a solution of 0.4 M HCl, 0.5 mg of protamine sulfate (grade X; Sigma) per ml, 8 M urea, and 0.02% Pyronine Y to the minichromosomes prior to electrophoresis (modified from reference 6). The gels contained 15% acrylamide (30:0.6 acrylamide/bisacrylamide), 6 M urea, 0.37% Triton X-100 (Sigma), and 5% acetic acid. The tube gels were prerun overnight at 200 V and 4°C and then subjected to 1 h of scavenging with 1 M cysteamine. The samples were run at 300 V in the cold. After this electrophoresis, the tube gels were equilibrated for 30 min to 1 h in several changes of 62.5 mM Tris (pH 6.8) until the pH remained at 6.8. For the last 15 min, β-mercaptoethanol (final concentration, 0.5%) was added to the equilibration buffer. The gel was then placed over an SDS–15% polyacrylamide gel, sealed with 1% agarose in 62.5 mM Tris (pH 6.8), and electrophoresed as described by Laemmli (32).

The marker histones were obtained from *X. laevis* erythrocytes. Erythrocyte nuclei were isolated as described by Humphries et al. (23), and the histones were extracted from the nuclei by the procedure of Adamson and Woodland (1). Phenylmethylsulfonyl fluoride (20 μg/ml), leupeptin (2 μg/ml), and pepstatin (2 μg/ml) were present in all buffers throughout the extraction; nevertheless, significant proteolysis of histone H3 was still observed (see Fig. 7c). All protein gels were stained with silver by the procedure of Wray et al. (71).

In vitro transcription reactions. To chromatin assembly reactions (see Fig. 1), a 1/10 volume of a mixture containing 6 mM each ATP, UTP, and CTP, 0.2 mM GTP (Pharmacia, Inc.), and 5 μCi of [α-^{32}P]GTP (Amersham Corp.) was added, and the reaction mixture was incubated at 27°C for 15 min.

Transcription with purified minichromosomes (see Fig. 8), in a total volume of 135 μl, was done with 100 ng of 5S DNA plasmid in the form of minichromosomes, without or with 100 ng of pXbs115/105 DNA (all samples made up to a final volume of 60 μl with 100 mM NaCl, 10 mM HEPES [pH 7.5], 0.2 mM EGTA, and 20% sucrose). A 60-μl volume of S-150 was added, and the mixture was made up to a final volume containing 100 mM NaCl, 20 mM HEPES (pH 7.5), 1 mM EGTA, 3 mM ATP, 7 mM $MgCl_2$, and 0.5 mM dithiothreitol. After appropriate incubation times at 27°C, as indicated in Fig. 8, nucleoside triphosphates plus [α-^{32}P]GTP were added for a 15-min pulse at 27°C.

The RNA was extracted from the reaction mixture and analyzed by electrophoresis in 8% polyacrylamide gels containing 8 M urea (62).

Indirect DNase I footprinting by primer extension. The indirect DNase I footprinting method is based on hybridizing a labeled primer to denatured DNA molecules and extending the primer with reverse transcriptase to the DNase I-created ends.

Between 200 and 300 ng of 5S DNA plasmid in the form of isolated minichromosomes was adjusted to 100 mM NaCl, 10 mM HEPES (pH 7.5), 0.2 mM EGTA, 0.5 mM dithiothreitol, and 5 mM $MgCl_2$ (kept on ice) and then digested with DNase I (Boehringer Mannheim) for 1 min at 23°C (final volume, 180 μl). The reaction was stopped by addition of 53 μl of 0.35% SDS, 70 mM EDTA, and 35 μg of tRNA per ml (from bakers' yeast; Boehringer Mannheim), and the mixture was digested with 0.3 mg of proteinase K per ml for 1 to 2 h. Following this, the nucleic acids were phenol extracted and ethanol precipitated.

The DNA was suspended in 10 μl of H_2O; 10 μl of 0.4 M NaOH and 0.4 mM EDTA was then added, and the DNA was melted at room temperature for 5 to 10 min. The denatured DNA was placed on ice and neutralized by addition of 3 μl of 3 M glacial acetic acid (pH 4.5); 80 μl of ethanol was added, and after this ethanol precipitation, the denatured DNA was washed again with 70% ethanol and dried.

The DNA pellet was dissolved in 10 μl of a mixture containing 50 mM Tris (pH 7.9), 8.5 mM $MgCl_2$, 40 mM KCl, and 2 pmol of the 5′ ^{32}P-labeled primer. The primer was annealed for 10 min at 50°C and briefly spun in a microcentrifuge, and 4 μl of a mixture containing 25 mM dithiothreitol, 0.75 mM each deoxynucleoside triphosphate (Pharmacia), 50 mM Tris (pH 7.9), 8.5 mM $MgCl_2$, and 40 mM KCl was added. After equilibration at 37°C for 2 to 3 min, 0.3 μg of single-stranded DNA-binding protein was added, and, following a further 10-min incubation, 6 U of AMV reverse transcriptase was added (U.S. Biochemical Corp.). The reactions were stopped after 30 min by phenol extraction. The labeled DNA was ethanol precipitated with 2.5 M ammonium acetate, washed with 70% ethanol, dried, and suspended in the formamide-dye mix. The labeled products were resolved in a 8% polyacrylamide sequencing gel containing 8 M urea.

The primers used are referred to as "reverse" and "universal" by the U.S. Biochemical Corp. The reverse primer hybridizes to a 16-base region on the coding strand, located on the pUC DNA 9 bases 5′ from the *Hin*dIII site of pXbsF201. The universal primer anneals to a 17-base region

on the noncoding strand located on the pUC DNA 14 bases 3′ from the *Bam*HI site (see Fig. 9). The primers were labeled at their 5′ ends with [α-^{32}P]ATP and T4 polynucleotide kinase.

RESULTS

Chromatin assembly is accompanied by repression of 5S RNA transcription. Chromatin assembly in the high-speed supernatant of *Xenopus* oocytes (oocyte S-150 [20]) is a complex, ATP-requiring process (20, 31, 50). Initial attempts in this laboratory to scale up the reaction failed. Recently (Shimamura et al., in press) we found that chromatin assembly is greatly enhanced by an ATP-regenerating system (maintaining an ATP concentration of 3 mM) and incubation at a higher temperature (37°C). Under these conditions, relatively large amounts of DNA are efficiently assembled into chromatin in convenient volumes (0.5 to 1 ml; see Materials and Methods). Moreover, this reaction is very reliable: more than 50 extracts have been prepared over the last year, and virtually all of them have been active and possess similar properties.

Oocyte S-150 also assembles the 5S RNA transcription complex, but the experimental conditions that favor chromatin formation lead to repression of 5S RNA transcription, and conditions that enhance transcription do not favor formation of chromatin (D. Tremethick and L. Millstein, unpublished observations). Under the experimental conditions chosen here, minichromosomes fully loaded with nucleosomes formed at 37°C, whereas maximal rates of 5S RNA transcription were observed at 27°C. We have therefore used 37°C incubations to assemble chromatin, and we have analyzed the transcriptional potential of the chromatin template by performing 15-min pulses of transcription at 27°C.

The plasmid used in these studies, pXbsF201 (47), carries 240 bp of somatic 5S DNA of *X. borealis* inserted into the 2,647-bp pUC DNA. Figure 1 shows the time course of DNA supercoiling (Fig. 1a) and transcription (Fig. 1b) when this plasmid was incubated in oocyte S-150 under the experimental conditions that favor chromatin assembly. The highest rate of transcription was observed after 15 min of incubation in oocyte S-150 at 37°C, at which time the relaxed DNA template had already become supercoiled. The rate of 5S RNA transcription decreased markedly at longer incubation times at 37°C, and after 3 h the 5S RNA transcription was barely detectable.

The interpretation of this experiment is not simple, because the incubation of S-150 at 37°C not only forms chromatin on the 5S DNA but also inactivates soluble transcription factors (as shown by the fact that extracts preincubated at 37°C without DNA lose transcriptional activity; D. Tremethick and A. Worcel, unpublished observations). It has been reported that the 5S RNA transcription complex, once formed, is stable to incubation temperatures as high as 37°C (67), and thus it is still possible that the factors bound to the 5S DNA in this experiment are more resistant to inactivation than the soluble factors are. In any event, to rule out the possibility that the observed inhibition in 5S RNA transcription is due solely to a 37°C inactivation of transcription factors and also to study the transcriptional capacity of minichromosomes formed at 37°C at different incubation times, we transferred the vials to 27°C and added fresh extract to them (Fig. 1c and d).

Under these conditions, chromatin assembly is inefficient at 27°C and does not appear to interfere with transcription

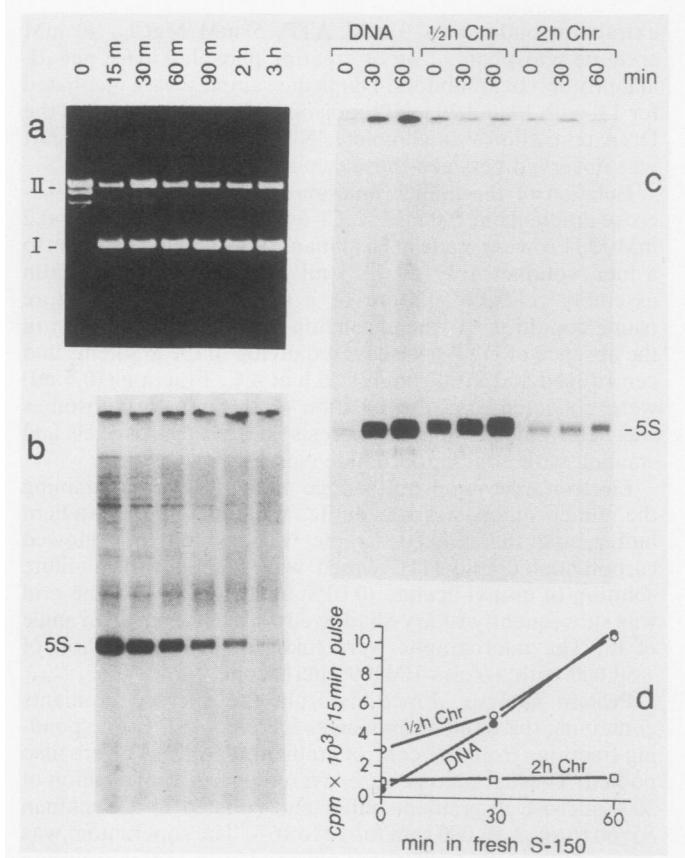

FIG. 1. Chromatin assembly and accompanying repression of 5S RNA transcription. Relaxed 5S DNA plasmid was assembled into chromatin in S-150 at 37°C under the standard conditions for the indicated times. (a) Half of the reaction mixture was processed to analyze DNA topology in a 1% agarose gel: I, supercoiled DNA; II, relaxed DNA. (b) To the other half of the reaction mixture, nucleoside triphosphates containing [α-^{32}P]GTP were added, transcription was allowed to proceed for 15 min at 27°C, and the reaction was processed for RNA analysis as described in Materials and Methods: 5S, 5S RNA. (c) In an independent experiment (with the same extract), chromatin was assembled in a total volume of 50 μl for either 30 min or 2 h as indicated; for the lanes marked DNA, the S-150 extract was replaced with extraction buffer, and the protein-free DNA was incubated at 37°C for 2 h. After the 37°C incubation, the vials were transferred to 27°C; 30 μl of fresh S-150 extract supplemented with 3 mM $MgCl_2$ was added, and the mixture was incubated for 0, 30, or 60 min at 27°C as indicated; following these incubations, transcription was assayed as before by a 15-min pulse. (d) The 5S RNA bands shown in panel c were excised and quantitated by liquid scintillation counting. The transcription rate attained after a 60-min incubation of DNA in fresh S-150 is equivalent to five transcripts per gene per hour.

(A. Shimamura and D. Tremethick, unpublished observations). Control 5S DNA incubated at 27°C in fresh S-150 is gradually assembled into transcription complexes, and the transcription rate increases during the first hour of incubation (Fig. 1c, left panel), after which a steady-state rate is achieved and maintained for about 3 to 4 h (data not shown). This type of kinetic phenomenon has been analyzed in detail by Bieker et al. (4), who showed that the lag in attaining maximal transcription rate is due to the slow formation of a rate-limiting complex of all the transcription factors and 5S DNA.

The 5S DNA minichromosomes assembled in S-150 for 30 min at 37°C were activated by fresh S-150 to the same extent

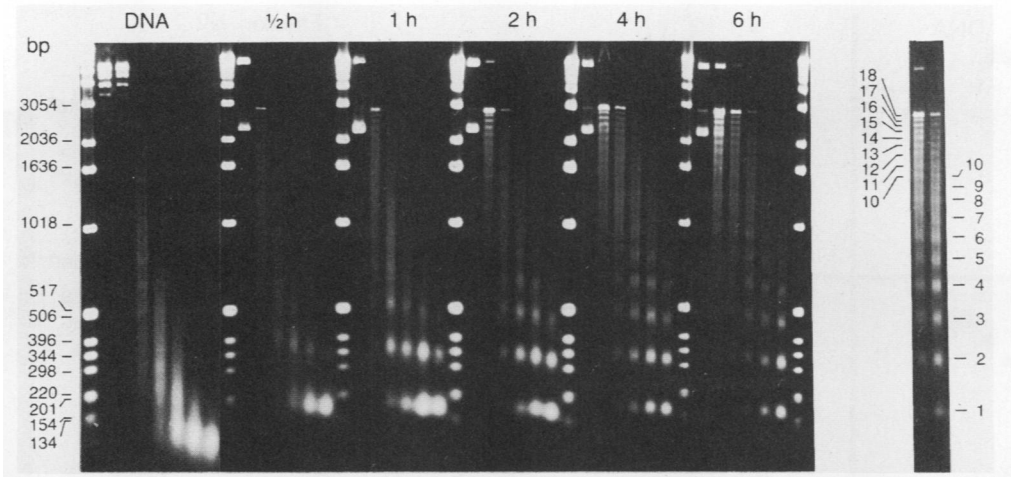

FIG. 2. Time course of chromatin assembly. Relaxed 5S DNA plasmid (2 μg) (shown in the second lane) was mixed with 400 μl of oocyte S-150 in a 700-μl final reaction volume under standard conditions (see Materials and Methods). Five identical 700-μl reaction mixtures were prepared and incubated at 37°C for 1/2, 1, 2, 4, and 6 h, as indicated. After the incubation, 300 μl was removed for the analysis of DNA topology as described in the legend to Fig. 3. To the remaining 400 μl, $CaCl_2$ and MNase (36 U) were added, and samples were removed at 0, 2, 4, 8, 16, and 32 min. For the protein-free DNA control, 1 μg of relaxed 5S DNA plasmid was placed in a 400-μl reaction mixture containing 3 mM ATP, 5 mM $MgCl_2$, and 40 mM creatine phosphate in extraction buffer; $CaCl_2$ and MNase (18 U) were then added, and samples were taken out at 0, 1, 2, 4, 8, and 16 min. The DNA samples were processed as described in Materials and Methods, and they were electrophoresed in 1% agarose gels with DNA molecular weight markers (Bethesda Research Laboratories, Inc.). The insert on the right displays the 4- and 8-min MNase digestion of the 6-h assembly product; the numbers 1 to 18 denote the nucleosome oligomers present in the partial digests.

as an equivalent amount of protein-free 5S DNA. In fact, at early times the transcription rate was higher in these minichromosomes than in free DNA, which is consistent with the notion that some factors have already bound to them during the first incubation at 37°C (Fig. 1c, center panel) (4).

In contrast, 5S DNA minichromosomes assembled for 2 h at 37°C were not readily activated by fresh oocyte S-150 (Fig. 1c, right panel). The rate of 5S RNA transcription on these minichromosomes, even after 1 h of incubation in fresh oocyte S-150 at 27°C, was only 1/10 the rate attained by the protein-free DNA under identical conditions (Fig. 1d). One possible explanation for the differences in the transcriptional capacity of early and late assembly products is that their structures differ. We examine this possibility below.

Structure of the chromatin formed after various times of incubation in S-150. Digestions with MNase after various times of incubation in S-150 reveal that nucleosomes were already present at 30 min but that a regular nucleosomal periodicity was not yet observed (Fig. 2). Both the amount of supercoiled DNA in oocyte S-150 and the amount of nucleosome-sized limit digest after MNase digestion reached maximum values at 1 h (Fig. 2, panel 3), at which time a 180-bp nucleosomal periodicity was seen (see Materials and Methods for the calibration of nucleosome repeat length; note in this panel the trinucleosome DNA band migrating above the 517-bp marker at early digestion times with MNase).

Beyond 1 h of continued incubation in S-150, the chromatin became more resistant to MNase, and the size of the nucleosome repeat decreased. This result indicates that more nucleosomes are being loaded on the DNA under these conditions. The length of the repeat decreased to about 170 bp at 2 h (Fig. 2, panel 4) and reached 160 bp at 6 h (Fig. 2, panel 6; note in this panel the trinucleosome DNA band migrating below the 506-bp marker at early digestion times with MNase). It should also be noted that the minichromosomes assembled for 6 h in oocyte S-150 generate an extended MNase ladder in which it is possible to count 18 regular steps between the linear DNA band and the mononucleosome DNA band (Fig. 2, insert).

Based on the measured length of the nucleosome repeat, the average total number of nucleosomes on this DNA is estimated to be 16 after 1 h of assembly (2,887 bp/180 bp per nucleosome repeat), 17 at 2 h (2,887/170), and 18 at 6 h (2,887/160). This last number is in agreement with the number of steps seen in the extended MNase ladder of Fig. 2.

Change in DNA linking number associated with nucleosome assembly. The relaxed DNA became supercoiled in oocyte S-150 within 15 to 30 min, and longer incubations did not change the DNA electrophoretic mobility in one-dimensional gels (Fig. 1 and 2). To resolve all of the DNA topoisomers, we performed gel electrophoresis in a second dimension in the presence of chloroquine (46) and matched the topoisomer distribution of the DNA samples against a standard carrying more than 20 DNA topoisomers (Fig. 3).

The supercoiled DNA topoisomers present after 30 min of incubation in S-150 displayed an average linking number change (ΔL) of -13. The negative linking number change increased at longer incubation times, reaching -16 at 2 h and -17 at 6 h. These results are consistent with the MNase digestion patterns shown in Fig. 2 and with the notion that additional nucleosomes are loaded on the DNA at longer incubation times in oocyte S-150.

Each nucleosome formed should introduce one negative linking number change in the covalently closed circular DNA (19, 57). The $-\Delta L$ determined here is slightly lower than the nucleosome number (n) estimated from the MNase digestion patterns. Thus, the average $-\Delta L$ at 2 h is 16 (Fig. 3, lower left panel), while the average number of nucleosomes (n) at 2 h is 17 (Fig. 2, panel 4). At 6 h $-\Delta L$ is 17 (Fig. 3, lower right panel), while n is 18 (Fig. 2, last panel). The same $-\Delta L/n$ ratio (0.95) was obtained in three experiments with two different extracts.

Purification of the in vitro-assembled minichromosomes.

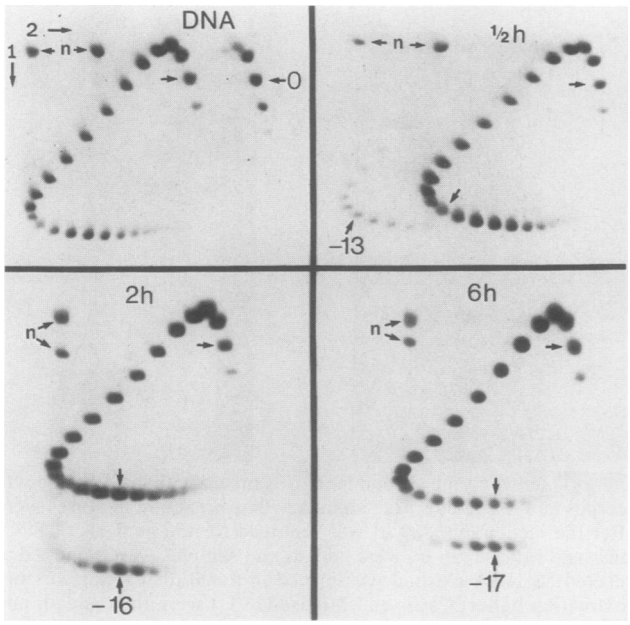

FIG. 3. Changes in DNA linking number (ΔL) during chromatin assembly. The remaining 300 μl from the chromatin assembly reactions (see legend to Fig. 2) was processed as described in Materials and Methods for two-dimensional gel analysis of DNA topoisomers. The DNA samples were coelectrophoresed with the standard DNA topoisomer ladder. To avoid overlap, the relaxed free DNA sample and the 30-min DNA sample (upper panels) were loaded at the same time as the standard, but several wells to the right (DNA) or to the left (30-min sample) from the standard; the 2- and 6-h samples (lower panels) were loaded in the same well as the standard, but they were allowed to enter the gel prior to the loading of the standard. The numbered outside arrow points to the center of the topoisomer distribution in each sample. The corresponding topoisomer in the standard (inside arrow) can be readily identified because it lies on a horizontal lane (upper panels) or vertical lane (lower panels) from the sample DNAs. The nicked DNAs (n) serve as additional reference points for the migration of DNA that is fully relaxed under these electrophoresis conditions. The chloroquine used in the second dimension unwound nine helical turns; an additional two helical turns were unwound, in both the first and second dimensions, because of differences in the ionic concentration between the incubation conditions and electrophoresis conditions (see Materials and Methods). The linking-number change in the minichromosomes (ΔL) can be readily determined by counting, in the topoisomer ladder, the number of steps from the relaxed DNA ($\Delta L = 0$) to the sample DNA.

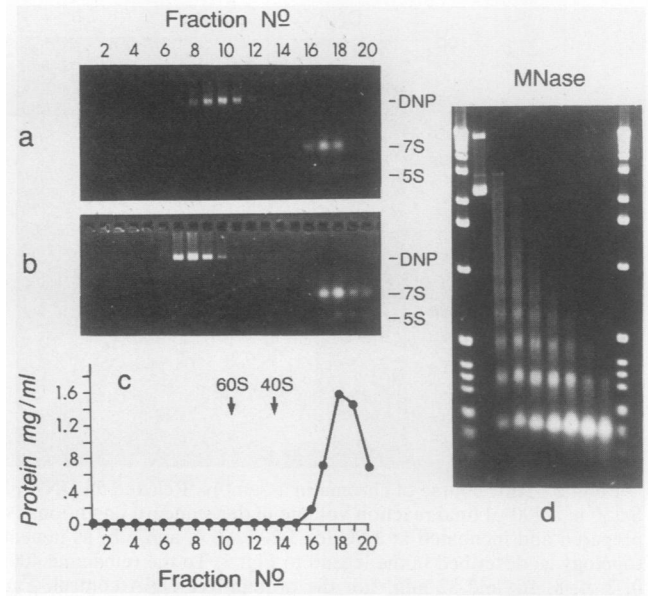

FIG. 4. Purification of the in vitro-assembled minichromosomes. Minichromosomes assembled in S-150 for either 30 min (a) or 2 h (b) were purified through sucrose gradients as described in Materials and Methods. After the centrifugation, fractions were collected and 20-μl portions of each fraction were electrophoresed in a 1.5% agarose gel to locate the position of the minichromosome (DNP) in the gradient. Abbreviations: 7S, TFIIIA-5S RNA complex; 5S, free 5S RNA present in S-150. The protein profile (c) was the same for the DNA-positive and for the DNA-negative control gradients; the arrows point to the position of isolated 40S and 60S ribosomal subunits that were centrifuged in a parallel sucrose gradient. Panel d displays the DNA fragments produced by an MNase digestion of the 2-h minichromosome performed, after the purification, as follows: to a 450-μl portion of the peak minichromosome fraction were added $CaCl_2$ and MNase (10 U), and 50-μl portions were removed at 0, 0.5, 1, 2, 4, 8, 16, and 32 min. The DNA samples were processed as described in Materials and Methods.

One approach to unraveling the in vitro processes of chromatin assembly and transcription complex formation is purification of the assembled minichromosomes and analyses of their properties. To this effect, we have used isotonic sucrose gradients (9, 69, 72), which avoid chromatin unfolding and aggregation and result in quantitative recovery of highly purified minichromosomes. Note that our protocols now call for a 2-h centrifugation of the oocytes at 150,000 × *g* instead of the original 30-min centrifugation of Glikin et al. (20). The extended centrifugation clears the 40S and 60S ribosomal subunits from the supernatant without significantly affecting its chromatin assembly capacity. The large amounts of ribosomes present in the oocyte and egg supernatants prepared in the past greatly hampered previous attempts to purify the small amounts of chromatin formed (see, e.g., references 35, 36, and 50).

By far the predominant fast-sedimenting structures present after DNA was incubated in the supernatants prepared by a 2-h spin at 150,000 × *g* were the compacted minichromosomes (Fig. 4). Furthermore, the sedimentation velocity of the assembled minichromosomes was a function of the number of nucleosomes that they contain: the 5S DNA minichromosomes sedimented at 60S after 30 min of assembly (Fig. 4a), at 70S after 2 h of assembly (Fig. 4b), and at 75S after 6 h of assembly (data not shown). The centrifugation through isotonic sucrose gradients did not alter the structure of the nucleoprotein: the isolated minichromosomes remained supercoiled and generated a digestion pattern with MNase that is indistinguishable from the one seen in unfractionated S-150 (Fig. 4d).

Figure 5 shows electron micrographs of minichromosomes isolated after 30 min, 2 h, or 6 h of assembly in S-150. The 30-min minichromosomes appear to be only partially loaded with nucleosomes, and free DNA stretches are clearly visible between nucleosomes; these minichromosomes display, on the average, about 10 to 12 nucleosomes. The 2-h minichromosomes display more nucleosomes, about 13 to 15, and much less free DNA than the 30-min minichromosomes do. The 6-h minichromosomes display very little free DNA and closely packed nucleosomes; about 14 to 16 nucleosomes can be visualized on the more favorably spread minichromosomes.

The assembled minichromosomes display a slightly smaller number of nucleosomes in the electron microscope than that estimated from the MNase digestion patterns and from

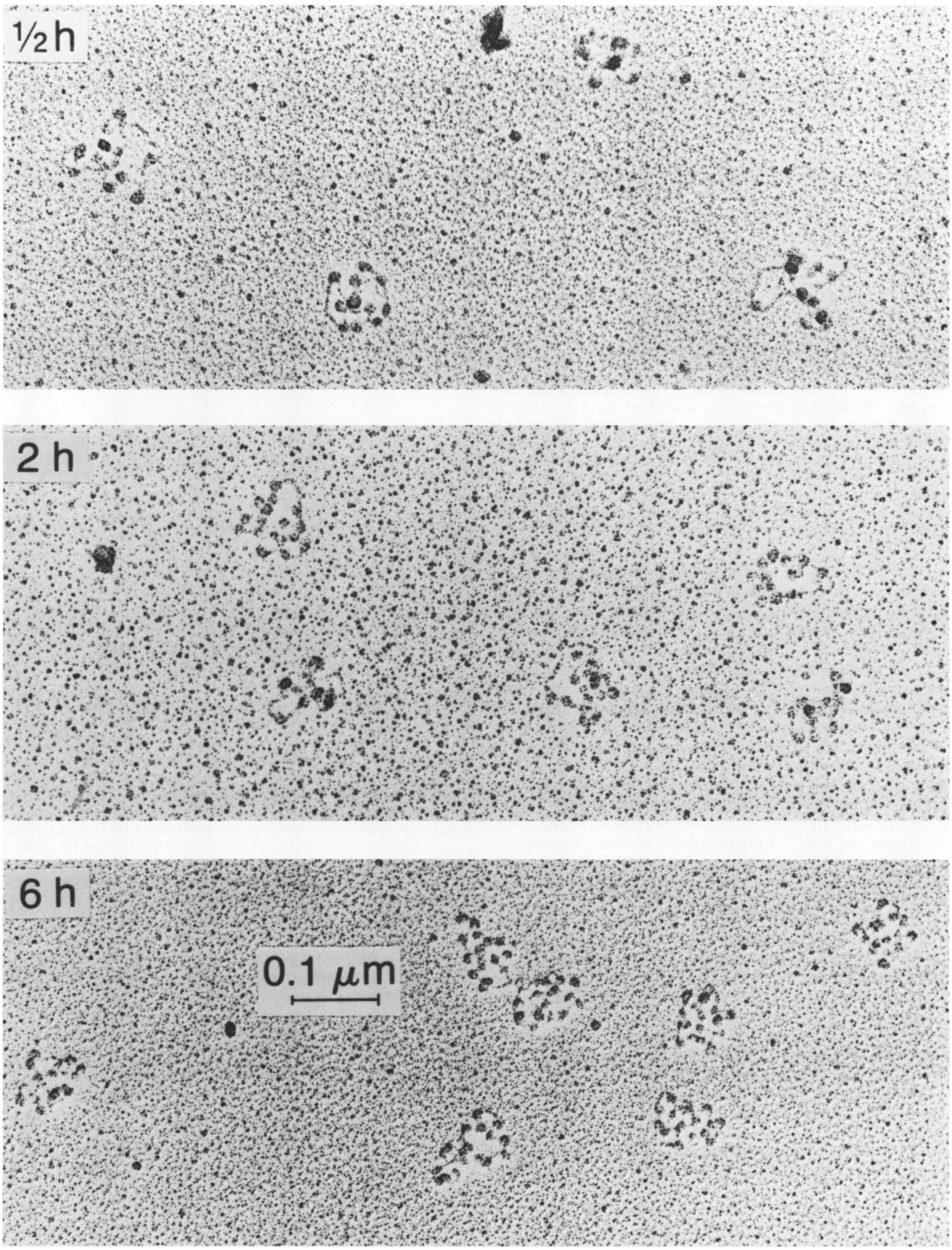

FIG. 5. Electron-microscopic visualization of the in vitro-assembled minichromosomes. Minichromosomes were assembled in S-150 for 30 min or 2 or 6 h as indicated. The minichromosomes were purified by sucrose gradient centrifugation as described in the legend to Fig. 4. Samples from the peak fractions were visualized by electron microscopy as described in Materials and Methods.

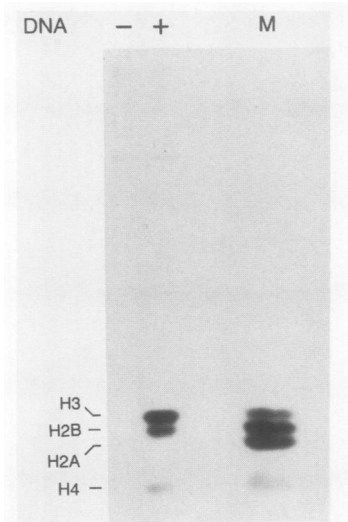

FIG. 6. Protein composition of the in vitro-assembled minichromosomes. Minichromosomes were assembled for 2 h in oocyte S-150 and purified by sucrose gradient centrifugation as described in the legend to Fig. 4. Mock-incubated oocyte extract (lane −) was also centrifuged through parallel gradients, and fractions sedimenting in the corresponding 50S to 80S position were pooled. The proteins were analyzed in SDS-polyacrylamide gels as described in Materials and Methods. M, Markers, nucleosomal histones of calf thymus.

the ΔL. The number of nucleosomes visualized with the electron microscope is probably an underestimate, because individual nucleosomes in these small and compacted minichromosomes cannot be readily distinguished from aggregated dinucleosomes. The minichromosomes, on the other hand, show no signs of aggregation, and, thus, their sedimentation velocity (Fig. 4) probably reflects their mass and their compaction ratio, as previously suggested.

Protein composition of the assembled minichromosomes. Each milliliter of oocyte S-150 contains about 2.5 mg of protein and assembles up to 5 μg of DNA into chromatin (see Materials and Methods). Thus, at the expected 1:1 DNA-histone stoichiometry of chromatin, a 500-fold purification is needed to separate the 5 μg of histone protein bound to the DNA from the total 2.5 mg of soluble protein. The sucrose gradient centrifugation (Fig. 4) achieves the required purification, as well as a very good recovery of the assembled chromatin.

The first two lanes in Fig. 6 display the total protein (after SDS-polyacrylamide gel electrophoresis [32]) present in the 50S to 80S fractions of the sucrose gradients of mock-incubated S-150 (lane −) and of S-150 incubated with DNA for 2 h (lane +). A barely detectable level of contaminating protein is seen in the high-molecular-weight range of this gel in both samples. The DNA-containing sample contains, in addition, a large amount of protein migrating in the molecular weight range of the nucleosomal histones.

The purification and the yield of histone are excellent, as expected, but the histone profile is rather unexpected. First, no histone H1 is seen associated with the assembled minichromosomes, and, second, the nucleosomal histones appear to be present in nonstoichiometric amounts in these SDS-gels.

The apparent deficiency in histone H2A in the SDS-gels was puzzling. This result prompted us to examine these proteins in Triton-acid-urea-gels, which give excellent resolution of the four nucleosomal histones and, particularly,

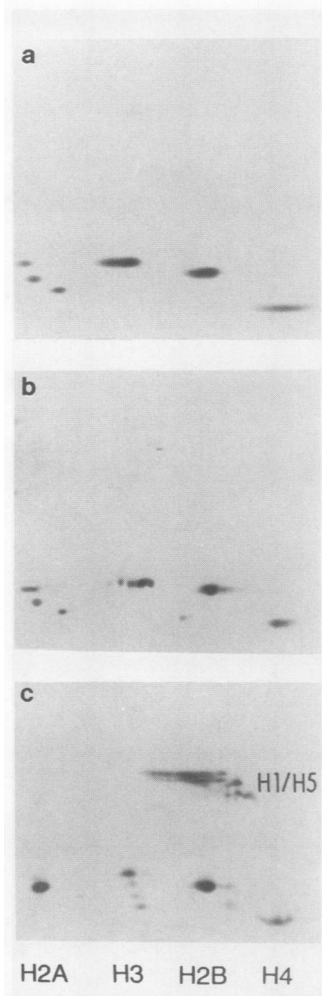

FIG. 7. Two-dimensional gel electrophoresis of the histones associated with the minichromosomes. Minichromosomes assembled in S-150 for 2 h (a) or 6 h (b) were purified as described in the legend to Fig. 6. Protein samples were electrophoresed in the first dimension in Triton-acid-urea-gels and in the second dimension in SDS-gels (see Materials and Methods). (c) Markers of *Xenopus* erythrocyte histones; the faint bands migrating below histone H3 in this gel are due to proteolysis of H3.

retard histone H2A to an easily distinguishable position (A. Zweidler and L. H. Cohen, Fed. Proc. Fed. Am. Soc. Exp. Biol. **31**:926A, 1972). These gels revealed that minichromosomes assembled for 2 h in oocyte S-150 contained three different H2A variants. The abnormal migration of two of the three H2A variants led them to be obscured by histones H3 and H2B in SDS-gels.

The protein composition of minichromosomes assembled for 6 h in oocyte S-150 (Fig. 7b) was similar to the one seen on the 2-h minichromosomes. The histone H2A variants we found in the in vitro-assembled minichromosomes were the same H2A variants recently identified in *Xenopus* egg extracts by Dilworth et al. (10). In addition, the nucleosomal histones in the assembled minichromosomes (Fig. 7a and b) appeared to be extensively modified, as indicated by the histone species of lower mobility present in the Triton-acid-urea dimension (compare with the pattern of the *Xenopus* erythrocyte histones shown in Fig. 7c). The two-dimensional gel electrophoresis also confirmed that histone H1 was not present in the assembled minichromosomes.

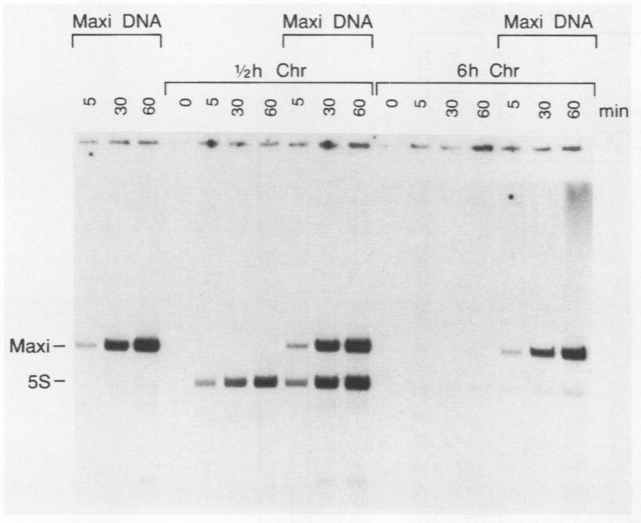

FIG. 8. Transcription in the S-150 of maxi-5S DNA and purified 5S DNA minichromosomes. 5S DNA minichromosomes were assembled in S-150 for either 30 min or 6 h as indicated and then purified through sucrose gradients as described in the legend to Fig. 4. Oocyte S-150 was added to the isolated minichromosomes, in the absence or presence of the protein-free maxi-5S DNA, as indicated. Reactions were incubated in the S-150 at 27°C for 5, 30, or 60 min, as shown; nucleoside triphosphates and [α-^{32}P]GTP were then added, and transcription was allowed to proceed for 15 min. In the 0 time lanes, minichromosomes were pulsed for 15 min as before, but in the absence of oocyte S-150. Abbreviations: Maxi, Maxi 5S RNA; 5S, 5S RNA. The bands were excised and quantitated as described in the legend to Fig. 3. The 30-min minichromosome attains the same rate of transcription as the coincubated protein-free maxi DNA; transcription in the 6-h minichromosome, on the other hand, is repressed 20- to 25-fold.

The assembled minichromosomes remain refractory to transcriptional activation. The absence of histone H1 in the minichromosomes was an unexpected finding, given the marked repression of transcription observed in oocyte S-150 (Fig. 1) and the probable role of this histone in gene repression (see, e.g., reference 65). Conceivably, histone H1 could have been bound originally to the DNA, but this extranucleosomal histone could have been preferentially lost afterwards, during the purification of the minichromosomes. We therefore assayed the transcription potential of the isolated minichromosomes to confirm that minichromosomes that do not contain histone H1 remain transcriptionally repressed in oocyte S-150.

In this series of experiments we used a "maxi"-5S RNA gene (5) as an internal control of the transcription potential of protein-free DNA, and we coincubated the protein-free DNA with the minichromosomes to prove that gene inactivation occurs only in *cis*. The maxigene was indeed active in oocyte S-150 at 27°C in all cases (Fig. 8). The minichromosomes isolated after 30 min of assembly were transcribed as efficiently as the protein-free maxigene, but minichromosomes isolated after 6 h of assembly were refractory to transcriptional activation in oocyte S-150 (Fig. 8). We have also observed a similar repression in minichromosomes isolated after 2 h of assembly (data not shown). As shown previously, minichromosomes isolated after 2 or 6 h of assembly did not contain histone H1 (Fig. 7a and b), and, therefore, the repressed state of the nucleoprotein must be due to factors other than histone H1.

Indirect footprint analyses show a phased nucleosome over the 5S RNA gene. To elucidate the reasons for the transcriptional repression of the 5S RNA gene in the assembled minichromsomes, we have used an indirect end label procedure to examine the structure of this gene in its chromatin configuration (see Materials and Methods). Briefly, minichromosomes were treated with DNase I, and the strand breaks were located by primer extension on the purified DNA. A similar technique has been used to footprint sequence-specific proteins bound to circular DNA (7, 21, 41) and to locate nuclease-hypersensitive sites in chromatin (64). The ability to assemble chromatin on a defined DNA circle greatly enhances the power of this technique; in addition, by performing the primer extension on the two DNA strands, it is possible to compare not only the location but also the frequency of cleavages occurring on one and on the other DNA strand in the same chromatin environment.

The resolving power of the indirect footprint of TFIIIA on the unlabeled DNA plasmid was as good as the one attained with end-labeled linear DNA fragments (Fig. 9, lanes 9 and 10 and lanes 17 and 18). Note on the coding strand (lanes 9 and 10) the hypersensitive site 5′ to the internal control region (ICR) and the complete protection over the ICR and, on the noncoding strand (lanes 17 and 18), the hypersensitive sites 3′ and 5′ to the ICR, as well as the site within the ICR (14).

The DNase I footprint on minichromosomes assembled in S-150 for 2 or 6 h is markedly different from either the protein-free DNA pattern or the TFIIIA footprint. A series of DNase I cuts spaced 10 bases apart is seen, and, between those cuts, DNA sites that were sensitive to DNase I on the protein-free DNA become resistant to DNase I in chromatin (Fig. 9).

On the coding strand (Fig. 9, lanes 1 to 5), the 10-base nucleosomal periodicity (44) extends to the center of the ICR, which indicates that a nucleosome is centered (phased) at or near the start site of transcription, as shown in the diagram in Fig. 9. Beyond the cut at +75, the chromatin pattern resembles the free DNA pattern up to position +250, where a 10-base nucleosomal periodicity is again detected. The position of the 5S RNA gene nucleosome is confirmed by the analysis of the DNase I cuts on the noncoding strand (see lanes 21 to 23), where a 10-base periodicity is seen extending from −60 to +60 on the 5S RNA gene. Again, 3′ to the +60 cut the chromatin pattern resembles the free DNA pattern. In contrast, 5′ to the −60 cut, another 10-base nucleosomal periodicity is detected starting at −90, which suggests that another phased nucleosome is located on the vector DNA next to the 5S RNA gene nucleosome (Fig. 9).

It should be noted that minichromosomes assembled in S-150 for only 30 min display a DNase I pattern that does not have the characteristic features of the chromatin pattern (see lanes 6 to 8 and lanes 19 and 20). Such minichromosomes already have about 13 nucleosomes (Fig. 2, 3, and 5), but those nascent nucleosomes are not yet uniquely phased on the DNA sequence. We are currently investigating the detailed features of the footprint over the 5S RNA gene at early times of chromatin assembly.

DISCUSSION

An in vitro system for chromatin assembly and transcription. Two modifications, described in Materials and Methods, have greatly improved the in vitro system for chromatin assembly from gently lysed *Xenopus* oocytes by Glikin et al. (20). One modification involves the incorporation of an ATP-regenerating system to maintain a constant ATP con-

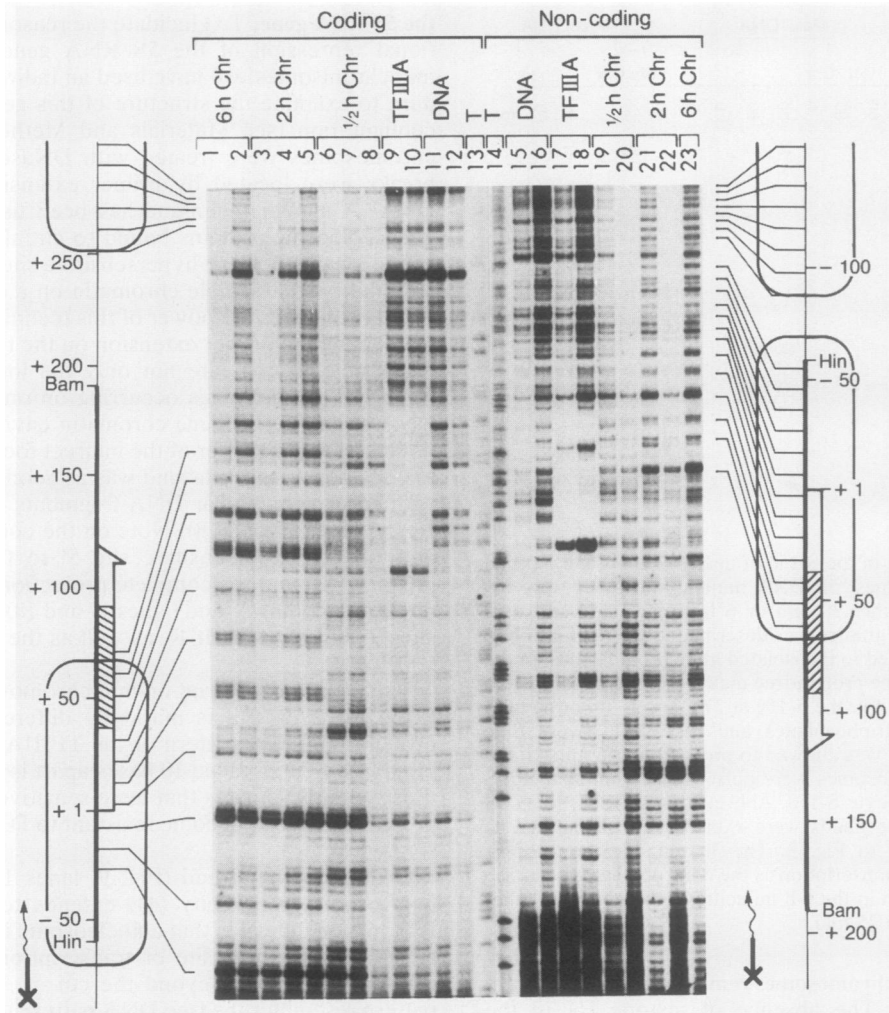

FIG. 9. Precise positioning of a nucleosome over the 5S RNA gene. The DNase I cleavages were mapped by a primer extension technique (see Materials and Methods). Abbreviations: T, dideoxy-adenosine sequencing ladder; DNA, protein-free 5S DNA plasmid; TFIIIA, TFIIIA-5S DNA plasmid complex; 1/2 hr Chr, 2 h Chr and 6 hr Chr, purified minichromosomes that were assembled in S-150 for 30 min, 2 h, and 6 h, respectively. The samples were digested as described in Materials and Methods with the following units of DNase I: lane 1, 20 U; lanes 2 and 23, 10 U; lanes 3 and 22, 24 U; lane 4, 12 U; lanes 5 and 21; 6 U; lanes 6 and 20, 9 U; lane 7, 3 U; lanes 8 and 19, 1 U; lanes 9, 11, 16, and 18, 4 U; lanes 10, 12, 15, and 17, 2 U. The outside maps show the 240-bp 5S DNA (▬) inserted in the pUC DNA (——), the 5S RNA gene (⟶), and the ICR (▨). Also shown in the diagrams are the positions of the phased 140-bp nucleosomes detected in the 2- and 6-h minichromosomes. The 10-base nucleosomal periodicity at the top of the gel in the coding strand is more clearly visible on a longer exposure of the autoradiograph. The short bar at the lower left in each diagram shows the size and position of the end-labeled pUC primers used for the DNA chain extension over the 5S DNA coding (left half panel) and noncoding (right half panel) template strands.

centration, 3 mM, throughout the incubation. As noted previously (20, 31), formation of regularly spaced nucleosomes in this extract requires ATP. We have found (A. Shimamura and A. Worcel, unpublished observations) that the added ATP is rapidly hydrolyzed during incubation in S-150 as described by Glikin et al. (20), and we suspect that variations in ATPase levels in different extracts may have contributed to the variability of the results obtained by previous methods.

The other modification is the temperature of incubation, 37°C, instead of the 24 to 27°C used by Glikin et al. (20). As was noted previously with other in vitro systems (48, 59, 60), nucleosomes form very efficiently at 37°C. The reasons for such a marked temperature dependence are not clear; perhaps the higher temperature further increases the solubility of the histones and favors their interactions with DNA. We have observed a similar enhancement of chromatin formation at lower temperatures when a lower pH (6.5 instead of 7.5) is used (Shimamura and Tremethick, unpublished data). Histones are more soluble at the lower pH, and the observed temperature effect may have a similar physical basis. Regardless of the mechanism, incubations at 37°C yield native chromatin in a reproducible manner and in amounts that are sufficient for proper chemical characterization. Because the reaction is very efficient at 37°C, chromatin with a high nucleosome density can be produced, and, by selecting an appropriate time, chromatin with the desired nucleosome spacing (e.g., 180 bp at 1 h and 160 bp at 6 h) can be achieved.

It should be noted that under slightly different experimental conditions, the same oocyte S-150 assembles the 5S RNA transcription complex (Fig. 1 and 8). The ease of preparation, the stability at −70°C, and the high rates of specific 5S RNA transcription attained with this extract also make it an excellent system for the study of 5S RNA gene expression in vitro (see, e.g., references 41, 42, 45, and 55). We stress,

however, that oocyte S-150 is a complex system for transcription because it also forms inactive chromatin on the 5S RNA gene, and, therefore, caution should be exercised when comparing results obtained under different experimental conditions.

Work in this laboratory has been directed toward the elucidation of the process of chromatin formation in S-150. To this effect, we have developed conditions for the quantitative assembly of plasmid DNAs into nucleosomal chromatin, and, under these conditions, the 5S RNA gene is inactivated and a phased nucleosome forms over the gene. On the other hand, under conditions that do not favor chromatin formation, a footprint of the endogenous TFIIIA is observed (42, 45). We have also observed TFIIIA footprints under such conditions. We know that the difference is not simply due to the higher incubation temperature that we used, which could in principle cause inactivation of the TFIIIA at 37°C, because a footprint of the endogenous TFIIIA can also be seen at 37°C under conditions of DNA excess that do not lead to the quantitative packaging of the plasmid onto nucleosomal chromatin (Tremethick and Worcel, unpublished observations).

Elucidation of the interplay between chromatin structure and transcription will require the use of in vitro systems, like the one described here, that couple chromatin assembly with transcription. This in vitro system should be particularly useful in examining the possible activities of proteins known to activate gene transcription in vivo in mammals and other higher eucaryotes (see, e.g., references 63 and 73). The required 37°C incubations, however, inactivate *Xenopus* transcription factors, and lower temperatures will have to be used to dissect the transcription apparatus of the *Xenopus* 5S RNA gene. It is possible to assemble chromatin with the oocyte S-150 at lower temperatures, but the reaction is not as efficient as at 37°C, and the process requires precise control of the Mg/ATP ratio as described by Knezetic and Luse (31). As previously reported, the nucleosomal repeat is longer when chromatin is assembled at lower temperatures (20, 50). Studies at lower temperatures will be presented elsewhere (A. Shimamura and A. Worcel, manuscript in preparation). We have limited the data here to chromatin formation at 37°C because this reaction is straightforward and we wished to focus this work on the nature of the repressed 5S DNA chromatin structure assembled in S-150.

How are nucleosomes spaced and phased on the DNA? Dilworth et al. (10) have shown that extracts from *Xenopus* eggs are deficient in histone H1, which probably explains why minichromosomes that are assembled in these extracts lack histone H1. Our data imply that the observed nucleosome spacing and phasing, and the accompanying repression of 5S RNA gene expression, must be a function, somehow, of the interactions between the nucleosomal histones and the DNA. The time-dependent changes observed in the structure and expression of the chromatin that forms in S-150 are particularly intriguing. Minichromosomes assembled for 30 min are about 70% loaded with nucleosomes, but a regular nucleosome periodicity is not seen and a nucleosome is not yet phased over the 5S RNA gene. These results suggest that nucleosomes are able to slide on this chromatin, and this may explain why such nascent nucleosomes can be displaced by a transcription complex (Fig. 1 and 8).

Minichromosomes assembled for 2 h in S-150 are fully loaded with nucleosomes and display a regular nucleosomal periodicity and a precisely phased nucleosome over the 5S RNA gene. These results indicate that the nucleoprotein is more rigid at 2 h; this is consistent with the observation that those static nucleosomes cannot be displaced by the 5S RNA transcription complex.

The data presented here on 5S RNA transcription with isolated minichromosomes as templates are in general agreement with the data of Knezetic and Luse (31) on RNA polymerase II transcription of minichromosomes templates. In that work, minichromosomes with different numbers of nucleosomes were produced in oocyte S-150 at 22°C by adjusting the ATP/Mg ratio, and those minichromosomes behaved in a manner similar to the one described here. No initiation of transcription by RNA polymerase II was observed on minichromosomes reconstituted with more than 70% of the normal nucleosome density, but those reconstituted with less than 33% of the normal nucleosome density transcribed as efficiently as free DNA.

We find that chromatin assembled with closely packed nucleosomes cannot be transcriptionally activated. The close packing of nucleosomes may prevent nucleosome movement and fix the chromatin, allowing for efficient repression in the absence of histone H1. This situation is analogous to the one observed in yeast chromatin, which lacks histone H1 (8) and has a short nucleosome spacing (160 bp [38, 61]). Higher eucaryotes, on the other hand, display longer nucleosome spacings and may require histone H1 for efficient repression. We are currently investigating transcriptional repression on minichromosomes assembled with different nucleosome spacings without and with exogenously added histone H1.

The present work does not address the question of the state of the histones in oocyte S-150 or of the mechanism by which DNA and eight protein molecules are assembled into one nucleosome. It is known that the histones stored in the *Xenopus* oocyte are extensively modified by negatively charged groups (68) and that histones H3 and H4 are bound to polypeptides N1 and N2 (25, 26) and histones H2A and H2B appear to be bound to nucleoplasmin (10). It is also known that in somatic cells new histones do not appear in the nascent chromatin simultaneously: histones H3 and H4 are detected first, then histones H2A and H2B, and, finally, histone H1 (54, 69). Further experiments should establish whether histone modifications and sequential histone deposition play a role in the maturation of the nucleosome and/or whether nucleoplasmin plays a direct role in the formation of the nucleosome, as proposed by Laskey and co-workers (10, 12, 33, 34).

Possible role of the phased nucleosome over the 5S RNA gene. The position adopted by the nucleosome covering the *Xenopus* 5S RNA gene in these minichromosomes is identical to the one seen when a nucleosome is reconstituted in the presence of counterions on a short linear DNA fragment carrying this sequence (48) or the sea urchin 5S RNA gene sequence (56). In this position, the start site of transcription is buried in the center of the nucleosome, in a region of strong interactions between histones and DNA (13), and the internal control region of the 5S RNA gene is located at one end of the nucleosomal DNA coil. In fact, TFIIIA can bind to this phased nucleosome, but the bound factor neither disrupts nor displaces the histone octamer (48). The histone octamer may prevent the binding of additional factors required for the assembly of the 5S RNA transcription complex, and this may explain why the minichromosomes are refractory to transcriptional activation in S-150. Because only a small fraction of 5S DNA molecules are active, the observed correlation between inhibition of 5S RNA transcription and nucleosome formation on the majority of the 5S DNA molecules does not signify that the nucleosome is

necessarily responsible for the inactivation of the 5S RNA transcription complex. However, the fact that minichromosomes assembled for 2 h cannot be reprogrammed for 5S RNA transcription (under the same conditions that protein-free DNA and 30-min minichromosomes are readily programmed [Fig. 1 and 8]) leads us to conclude that the phased nucleosome observed over the 5S RNA gene on the 2-h minichromosomes is probably responsible for making these minichromosomes refractory to transcriptional activation.

Our results are consistent with the work of Lassar et al. (37), showing that this 5S RNA gene in a simian virus 40 minichromosome is refractory to transcription by RNA polymerase III in the absence or presence of the other transcription factors. The repression they observed also appeared to be independent of histone H1, because the block was not relieved by sedimenting the minichromosomes through 0.6 M NaCl. The association of histone H1 with this inactive chromatin and the subsequent condensation of the nucleoprotein may provide an additional level of repression (see, e.g., references 53 and 65).

Nucleosomes that are precisely positioned over promoter regions have been found not only in constitutive genes such as the 5S RNA gene but also in inducible genes, from yeasts to mammals. Examples are the low-phosphate-inducible promoter of the *PHO5* gene in *S. cerevisiae* (3) and the steroid-inducible mouse mammary tumor virus promoter (49). These phased nucleosomes are displaced or disrupted on induction, and this exposes additional upstream activating DNA elements.

Eucaryotic organisms repress gene expression in a stable and efficient manner, and histones probably play a key role in this gene inactivation. Histones do not appear to be required, in general, for transcription. Thus, histone-free linear 5S DNA fragments are transcribed at a high rate in vitro (67), and a block in histone H4 production in *S. cerevisiae* does not inhibit transcription in vivo; in fact, the *PHO5* gene and other inducible genes are no longer repressed in the histone H4-deficient cells (21a). Clearly, a better knowledge of the mechanism of nucleosome formation should help elucidate the processes that nature has evolved to disrupt, or prevent the formation of, a phased nucleosome over a key regulatory sequence.

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