Pathways of nucleoprotein assembly on 5S RNA genes in a *Xenopus* oocyte S-150 extract

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Received February 7, 1989; Revised and Accepted April 28, 1989

ABSTRACT

Conditions for transcription and nucleosome assembly of plasmids bearing *Xenopus* 5S RNA genes have been monitored in the whole oocyte S-150 extract (1). We find that the optimal conditions for transcription differ substantially from optimal conditions for nucleosome assembly. DNA molecules bearing as few as 50% of the native density of nucleosomes are transcriptionally inert. Although the 5S gene-specific transcription factor TFIIIA is in excess in this extract, these nucleosome reconstitutes do not exhibit TFIIIA-like DNase footprints nor do these reconstitutes bind exogenous TFIIIA. We have also examined the nucleotide requirement for DNA supercoiling and for generation of 5S gene transcription complexes. Supercoiling associated with nucleosome assembly does not require ATP; however, nucleotide hydrolysis is required for establishment of active complexes. Phosphorylation of a 200 kdalton protein occurs in a 5S DNA-dependent manner concurrent with the generation of primed transcription complexes. Results of nondenaturing gel electrophoresis coupled with a second dimension of SDS gel electrophoresis suggest that the 200 kD protein may be a component of the 5S RNA gene transcription complex.

INTRODUCTION

Transcription of the 5S RNA genes of the frog *Xenopus* has been a subject of intensive study over the past decade (for reviews, see refs. 2,3). Transcription *in vitro* requires at least three transacting protein factors in addition to RNA polymerase III (4,5). One of these protein factors, transcription factor IIIA (TFIIIA), has been purified to homogeneity and has been shown to bind the internal control region of the gene (6,7). This protein has been studied extensively with respect to both gene (8,9) and protein structure (10,11). The two other protein fractions required for transcription, TFIIIB and TFIIIC, interact with the 5S gene-TFIIIA complex to form a stable transcription complex (5,12). This complex persists through many cycles of transcription and is thought to be a major determinant of the developmental regulation of the 5S gene families (3). As genes in the eukaryotic cell nucleus exist as chromatin rather than protein-free DNA, several laboratories have begun to study the influence of nucleosome structure on the transcriptional activity of the 5S RNA genes (1,13–18) and the effect of nucleosome reconstitution on TFIIIA binding (19,20).

In studies with whole oocyte S-150 extracts reported by Worcel and his colleagues (1,17,18), a correlation between DNA supercoiling or gyration and 5S gene transcriptional activity was noted, although other studies have called these results into question (21,22, this study). Wolffe et al. (21) have argued that 5S transcription can be obtained *in vitro* with nuclear extracts which do not supercoil DNA and with short linear templates. Nonetheless, it seemed pertinent to examine in detail the relationship between chromatin

assembly and 5S gene transcription in the oocyte S-150 extract. We find that the optimal conditions for chromatin assembly and transcriptional activity are quite different and that nucleosome reconstitution on a 5S gene in the S-150 precludes TFIIIA binding and transcription. We also explore the nature of the nucleotide requirement for establishment of a transcription complex in the S-150.

MATERIALS AND METHODS

Preparation of extracts and transcription reaction conditions

S-150 extracts were prepared from the oocytes of mature *X. laevis* females essentially as described (1,23,24) with the following modifications. After collagenase treatment the large stage V and VI oocytes were washed extensively in OR-2 medium and collected by gravity sedimentation on nytex filters. Approximately 200ml of OR-2 was used per wash and the oocytes were washed at least 10 times in OR-2 and 2 times in homogenization buffer (24) prior to centrifugation at 150,000g for 30 min in the SW50.1 rotor. Only the clear region of the extract was collected by puncture of the centrifuge tube with an 18 gauge hypodermic syringe needle. Aliquots were immediately frozen at -70°C . Transcription reactions contained in a final volume of 50 μl the amounts of DNA indicated in the figures and text, 30 μl of the S-150 extract, glycerol to 8% (v/v), MgCl_2 to 6 mM, UTP and CTP at 600 μM , and GTP at 40 μM . Reactions also contained 10 μCi of ^{32}P -GTP. ATP was included at 600 μM for standard transcription reactions. These reaction conditions are referred to as transcription conditions. Some experiments contained higher levels (1 to 3 mM) of ATP as indicated in the text and figure captions. Plasmid DNA was linearized with restriction enzyme Pst I or relaxed with DNA topoisomerase I (Bethesda Research Labs). DNA was purified from these incubations by proteinase K digestion (200 $\mu\text{g}/\text{ml}$, 37° , 1h) and phenol extraction and ethanol precipitation.

Analysis of DNA topology in the S-150

The 883 bp Hind III insert in plasmid pXls11 containing the 5S gene was isolated after restriction of the plasmid DNA and polyacrylamide gel electrophoresis. This DNA was labeled by nick translation using the random primer method (Pharmacia Oligo labeling kit). The DNA was labeled to a specific activity of greater than 1×10^8 cpm per μg . DNA was purified from incubations in the S-150 by incubation with proteinase K (200 $\mu\text{g}/\text{ml}$) and SDS (0.5%) for at least 30 min at 42°C and repeated extractions with phenol-chloroform followed by ethanol precipitation. DNA was analyzed on 1% (w/v) agarose gels in 44 mM Tris-borate, 1 mM EDTA, pH 8.3, buffer. The DNA was denatured in the gel and transferred onto nitrocellulose and hybridized with the labeled probe using standard methods.

DNase Footprinting

The 883 bp Hind III fragment of pXls11 was dephosphorylated with calf alkaline phosphatase and phosphorylated with [γ - ^{32}P]ATP and polynucleotide kinase. This DNA was then cyclized with T4 DNA ligase as described (20). Labeled ends of linear molecules were dephosphorylated such that the only remaining labeled DNA was circular. These samples contained approximately 75% monomer circles and 25% dimers, trimers and tetramer length circles. TFIIIA was prepared from immature oocytes by standard procedures employing glycerol gradient centrifugation to isolate 7S RNP particles, DEAE cellulose chromatography, RNase digestion and Bio-Rex 70 chromatography (24). The protein was greater than 95% homogeneous as judged by SDS polyacrylamide gel electrophoresis and silver staining. After incubation with TFIIIA (20,24) or in the S-150, MgCl_2 was adjusted

to 5 mM, and the samples were digested with DNase I at 0.2 to 0.5 $\mu\text{g/ml}$ for deproteinized DNA or TFIIIA-DNA complexes or with 2 to 5 $\mu\text{g/ml}$ for S-150 samples. Incubations were for 30 sec to 5 min at 22°C. Reactions were stopped by the addition of SDS to 0.5% and EDTA to 50mM and DNA was purified from these digests with proteinase K (200 $\mu\text{g/ml}$ for 1 h at 37°C) and phenol extractions. This DNA was sequentially cleaved with Hind III and Taq I. The latter enzyme cleaves the linear Hind III fragment 25 bp from the left hand ^{32}P -labeled Hind III end, thus leaving the remaining molecule with a unique end label on the coding strand, 160 bp from the 3' end of the 5S gene. The resulting DNA was phenol extracted, ethanol precipitated, dissolved in formamide, heated to 95°C for 2 to 5 min, and analyzed by gel electrophoresis under denaturing conditions (50%, w/v, urea) on thin sequencing gels. Autoradio-graphic exposures were obtained with Kodak XAR film at -70°C with Cronex intensifying screens. Samples with comparable extents of digestion are shown in Figure 4.

Labeling of oocyte proteins and analysis

30 μl aliquots of S-150 extract were incubated with or without added DNA (500 ng) with 6 mM MgCl_2 , 1 mM ATP and 10 μCi of $\gamma^{32}\text{P}$ -ATP (3000 Ci/mmmole, NEN). After 3h at 22°C, the samples were chromatographed directly on 1 ml columns of Sepharose 6B-CL equilibrated in 60 mM KCl, 20 mM Hepes, pH 7.5, 5 mM MgCl_2 , 10% glycerol. Columns were packed in 1 ml disposable syringes plugged with siliconized glass wool and run by gravity flow. One ml fractions were collected and to the void fractions containing DNA and associated proteins trichloroacetic acid was added to 25% (w/v) by the addition of 0.33 ml of 100% (w/v) TCA. After 1 h on ice, precipitates were collected by centrifugation at 12,000g for 15 min and washed extensively with ethanol to remove TCA. Samples were dissolved in SDS sample buffer, boiled and subjected to SDS-polyacrylamide gel electrophoresis. Autoradiographic exposures were made with Kodak XAR-5 film at -70°.

Nondenaturing gel electrophoresis

A 248 bp fragment containing the *X.borealis* somatic-type 5S gene was obtained by digestion of plasmid pXp10 (25) with Bam H1. An aliquot of this DNA was dephosphorylated and labeled with polynucleotide kinase and $\gamma^{32}\text{P}$ -ATP. 0.5 μg of Bam H1-digested plasmid DNA (vector plus insert) with or without an aliquot of the labeled fragment was incubated with 30 μl of S-150 as described above. Glycerol was added to the sample to a final concentration of 10% (v/v) and the sample was subjected to electrophoresis in a 5% (29:1 acrylamide to bisacrylamide) gel containing 44 mM Tris-borate, pH 8.3. The gel was pre-electrophoresed for 1 h prior to electrophoresis at 135 V for 3.5 h. The gel measured 0.75 mm by 20 cm. Western blotting and probing with anti-TFIIIA polyclonal serum (described in detail by Blanco et al., submitted) was performed using standard procedures. TFIIIA was isolated as described above and was subjected to SDS gel electrophoresis and the gel slice containing TFIIIA was crushed and used for rabbit immunization. The antiserum was used at a dilution of 1:500 with filters blocked with nonfat dry milk. Positive antibody reaction was detected with the Promega Protoblot system (alkaline phosphatase conjugated goat anti-rabbit antibody). For second dimension SDS gels, a gel slice containing ^{32}P -labeled proteins (see above) was excised from the nondenaturing gel (after a brief autoradiographic exposure) and equilibrated for 30 min at 20°C in a buffer containing 0.125 M Tris, pH 6.8, 0.1% SDS, 10 mM 2-mercaptoethanol and 10% glycerol. The gel slice was placed between glass plates directly above the stacking gel of a 12% SDS gel. Autoradiographic exposures were obtained with XAR5 film at -70°C.

Figure 1. Topology of the input DNA influences transcriptional activity. Plasmid DNA was either linearized with Pst I or relaxed with DNA topoisomerase I. DNA purified after these incubations was tested for transcriptional activity with the preincubation times indicated in the figure. 500 ng of the indicated DNA was preincubated in 30 μ l aliquots of S-150 containing 6 mM $MgCl_2$ and 3 mM ATP. After the indicated times, the remaining unlabeled and labeled nucleoside triphosphates were added and transcription was allowed to proceed for 2h prior to isolation of RNA and analysis by denaturing polyacrylamide gel electrophoresis. The autoradiogram is shown.

RESULTS

Transcriptional activity of the oocyte S-150

The transcriptional activity of the *Xenopus* somatic-type 5S gene contained in plasmid pXls11 has been monitored with a variety of extracts prepared from immature oocytes, mature oocytes, oocyte nuclei, unfertilized eggs and developing embryos (23–27). Using our standard conditions of 100 ng of gene-containing plasmid and supplementing the reaction with vector DNA to the DNA optimum determined for the individual extract (generally 500–600 ng), we find a maximal rate of synthesis of 7.5 transcripts per gene per hour (TGH) for the oocyte S-150 (1) and 15 TGH for the oocyte nuclear extract (25). Reducing the quantity of gene containing plasmid while maintaining the total DNA concentration at the optimum determined for the extract has the effect of increasing the number of transcripts per gene ('rate enhancement', 25). For the oocyte S-150 we have achieved a rate of approximately 50 to 75 TGH at 5 ng of gene plasmid in a 50 μ l reaction. This value is about one-third of the level of transcription observed *in vivo* in microinjected oocytes (28). However, most oocyte S-150 extracts we have prepared yield approximately 1 to 2 TGH under our standard conditions. This variability in transcription rates observed with different extracts is due in part to the concentration of immature oocyte-type TFIIIA in extracts prepared from different individual frogs (27).

Figure 2. Effect of preincubation time on DNA topology and transcriptional activity in the S-150. 5 ng of pXls11 and 500 ng of pUC19 DNA were preincubated in 30 μ l of S-150 extract with MgCl_2 added to 6 mM. Preincubation times are indicated in the figure. Equivalent reactions also contained 3 mM ATP, as indicated. Panel A shows a Southern blot of an agarose gel of DNA purified from these incubations probed with a 5S gene-specific ^{32}P -labeled restriction fragment derived from plasmid pXls 11. The positions of supercoiled (form I), relaxed and nicked circles (forms I_r and II), as well as dimer molecules, in the original input DNA are indicated. Panel B shows the products of transcription reactions with similar aliquots of the preincubation mixtures incubated for an additional 2 h under transcription conditions.

We have monitored the kinetics of transcription in different S-150 extracts and find quite different results with different extracts. Those extracts which yield high rates of transcription (7.5 TGH) exhibit a 20–30 min lag before attaining maximal rates of synthesis; the reaction is then linear for at least 4 h. This lag has been interpreted as the time required for the assembly of active transcription complexes (5,30). Similar results have been reported for the oocyte nuclear extract (25,26,29) and the HeLa S-100 (5,30,31). Most S-150 extracts we have examined, however, exhibit rather unusual preincubation kinetics. In the experiment of Figure 1, we monitored the transcription of somatic-type 5S DNA after various times of pre-incubation in the S-150 in the presence of 6 mM MgCl_2 and 1 mM ATP. Transcription was initiated by the addition of the other unlabeled and labeled nucleoside triphosphates. After an additional 2 h incubation, RNA was isolated and analyzed by gel electrophoresis. Figure 1 shows preincubation experiments for input DNAs linearized by the action of the restriction enzyme PstI, DNA relaxed by the action of DNA topoisomerase I and supercoiled DNA (at bacterial superhelix density). Linear DNA is a poor substrate for 5S transcription in the S-150 extract yielding only 2–5% of the transcripts obtained with circular DNAs; this contrasts with high level transcription of linear DNA obtained with the oocyte nuclear extract (25) and the HeLa S-100 (31). Relaxed and supercoiled DNA are equally active, but relaxed DNA requires a shorter preincubation time to attain maximal transcription rates (1.5 to 2.5 h versus 3.5 h for supercoiled DNA). Curiously, both templates lose activity on long times of incubation in the S-150 prior to initiation of transcription. We do not know the reason for the difference in optimum preincubation times for relaxed and supercoiled input DNAs.

Figure 3. DNA supercoiling and transcription under conditions for optimal supercoiling. 5 ng of pXIs 11 DNA and 100 ng of pUC19 DNA were incubated for the indicated times with 30 μ l of S-150 containing 6mM MgCl_2 and 3mM ATP. Panel A shows the Southern blot of an agarose gel of DNA purified from these incubations probed with labeled 5S DNA. Panel B shows the products of transcription assayed after the preincubation times given in panel A. Transcription was initiated by the addition of labeled and unlabeled nucleoside triphosphates and terminated after an additional 2h. Panel C shows comparable reactions to those in panel B with incubations entirely under transcription conditions as described in Materials and Methods. Labeled GTP was included in the last 2h of incubation.

We wondered whether the preincubation time optima could represent changes in the topological state of the DNA template brought about by topoisomerases or chromatin assembly in the S-150 (1). To examine the effect of preincubation in the S-150 on DNA topology, we analyzed the 5S gene-containing plasmid by agarose gel electrophoresis and Southern blotting (Figure 2A). Ideally, we wish to detect only those 5S DNA molecules which serve as a template for transcription. In order to achieve this, we decreased the amount of 5S plasmid DNA to 5 ng per reaction and maintained the total DNA concentration at 500 ng with vector pUC19 DNA. 500 ng per 50 μ l reaction is the DNA optimum for transcription determined for the extract used in this experiment (data not shown). These conditions give maximal rates of 5S synthesis on a per gene basis (50 TGH) and thus maximal amounts of the input DNA serve as templates for transcription (25). From the present data, however, it is not possible to directly assess the fraction of active molecules. Nonetheless, all molecules are bound by TFIIIA under these conditions (23–24) and are

assembled in large protein-DNA complexes (see below). The 5S gene plasmid is detected by Southern blot hybridization of the agarose gel using a 5S gene-specific hybridization probe. Panel A shows the distribution of 5S gene plasmid DNA in the agarose gel after different times of preincubation. In agreement with previous studies, the DNA is initially relaxed in a rapid reaction and then acquires supercoils in a time dependant fashion (32,1). We find, however, that changes in DNA topology as a function of preincubation time do not correlate with changes in transcriptional activity monitored in a subsequent 2h incubation (compare panels A and B). In the experiment depicted in Figure 2B, plasmid DNA containing the somatic-type 5S RNA gene was preincubated for the indicated times with the S-150 extract along with 6 mM $MgCl_2$ and, in duplicate reactions with 3 mM ATP, and transcription was initiated by the addition of the other unlabeled nucleoside triphosphates and labeled GTP. An optimal preincubation time of approximately 1 to 2 hours was observed with or without added ATP in this experiment. The exact preincubation time required for optimal levels of transcription has been found to vary between different extracts (compare Figs. 1 and 2B). Longer times of preincubation resulted in largely inactive templates (see below). Similar results have been obtained with other genes transcribed by RNA polymerase III, including the oocyte-type 5S gene, a *Xenopus* tRNA^{met} gene and the *Xenopus* OAX repetitive element (data not shown). This phenomenon, therefore, is not restricted to the somatic 5S gene.

The reintroduction of supercoils correlates with the inactivation of transcription seen on long times of preincubation (3 to 4h, lanes 11 to 14). It is noteworthy that the DNA template incubated in the S-150 for 15 min in the absence of all four nucleoside triphosphates is vastly less active in transcription than the same sample without preincubation (compare lanes 1 and 3 of panel B). At present we do not have an explanation for this phenomenon. We do not observe any pronounced effect of added ATP on supercoiling and only a small stimulation of transcription with added ATP in this experiment (but see below). To directly assess the role of ATP in the introduction of supercoils, we depleted the S-150 of endogenous ATP by the action of hexokinase (0.1 unit per 30 μ l reaction) and dextrose (3 mM) for 40 min prior to addition of DNA. In parallel reactions ATP was added back to 3 mM and the time course of supercoiling was monitored. We found no effect of ATP depletion on either the extent of introduction of supercoils or the time required to introduce supercoils (data not shown).

We also find that supercoiling and transcription have different DNA optima, with the optimum for maximal supercoiling at a far lower input of DNA than for transcription (approximately 100 ng per 30 μ l of S-150). Figure 3 shows the result of a comparable experiment to that depicted in Figure 2 but at the lower DNA input (100 ng of total DNA and 5 ng of gene containing plasmid). Under these conditions, a greater extent of supercoiling is attained. We interpret the introduction of supercoils as a measure of chromatin assembly (32–33). From comparisons of micrococcal nuclease digestion profiles, EM visualization, and changes in supercoiling, it has been established that assembly of one nucleosome on a DNA plasmid results in the reduction of the linking number of the plasmid by a value of 1 (upon the action of DNA topoisomerases) (33). It is likely that 500 ng of DNA far exceeds the available histone pool in 30 μ l of S-150. With 100 ng of total DNA in 30 μ l of S-150 extract, the DNA attains an average linking number which represents 50–75% of the bacterial superhelical density (data not shown). This corresponds to an average nucleosome density of 50–75% that of cellular chromatin (33). After the indicated preincubation times, the additional unlabeled and labeled nucleoside triphosphates

Figure 4. DNase footprint analysis in the S-150. Lane 1, DNA, no digestion; lane 2, protein-free DNA; lane 3, TFIIIA-DNA complex; lane 4, labeled DNA plus 100 ng pUC19 DNA incubated in 30 μ l of S-150 under the conditions of Figure 3 for 3 h prior to DNase digestion; lane 5, as in lane 4 but with 100 ng TFIIIA added 20 min prior to DNase digestion. M, Hpa II fragments of pXls 11 DNA. The arrow denotes the location of the 5S RNA gene and direction of transcription; the TFIIIA binding site is also indicated.

were added and the products of transcription were analyzed after an additional 2 h incubation (Figure 3, panel B). For comparison, panel C shows the products of transcription obtained with the same extract for comparable incubation times entirely under transcription conditions (yielding 50 TGH, but with labeled GTP for only the last 2 h of incubation). Clearly, conditions for chromatin assembly do not support active 5S transcription. Transcription conditions produce 10 to 20 fold more 5S RNA transcripts per unit time than optimal supercoiling conditions. At low levels of transcription we often note shorter than full length 5S RNA transcripts (Fig. 3B, lanes 1–2).

We have employed DNase footprinting in order to monitor the binding of TFIIIA to the 5S gene internal control region during incubation in the S-150. We previously showed that under transcription conditions TFIIIA binding could be detected on both linear and circular DNA molecules incubated in the S-150 (23,24). Figure 4 shows the results of footprint analysis on an internally labeled 883 bp circular DNA bearing the somatic-type 5S gene. This DNA was used rather than linear restriction fragments since linear DNA is a poor template for transcription in the S-150 (Fig. 1). Further, the 883 bp circle has

the same transcriptional properties as plasmid DNA harboring this 883 bp insert. The expected TFIIIA footprint is observed with pure DNA and protein (lane 3); however, no TFIIIA footprint is observed on this DNA after a 3 h incubation in the S-150 under conditions determined for maximal supercoiling (Figure 3). The pattern of DNase cleavages observed under these conditions (lane 4) is reminiscent of nucleosome reconstitutes formed with purified histones on this DNA (20). The regular repeating pattern of DNase-cleaved and protected regions with a periodicity of multiples of 10 nucleotides is diagnostic of a positioned nucleosome. However, the relative intensities of nucleosome-specific and naked DNA-like cleavages suggest that a significant fraction (perhaps 20%) of the molecules lack positioned nucleosomes. We also wished to determine whether TFIIIA could bind to this DNA after incubation in the S-150. To this end, we added a large excess of purified TFIIIA prior to DNase digestion. Even at a 100 fold excess of TFIIIA over DNA binding sites, no characteristic TFIIIA footprint was observed. This suggests that TFIIIA cannot bind the chromatin reconstitutes formed in the S-150. This result is in agreement with results obtained with reconstitutes formed with purified histones and restriction fragments from the *X. laevis* somatic-type gene (20). TFIIIA binding has been noted, however, with reconstitutes formed on short restriction fragments of the *X. borealis* 5S gene (19) and on similarly sized restriction fragments containing the *X. laevis* 5S gene. In this situation, TFIIIA may partially displace the histones from the DNA and bind itself (19).

Role of ATP in the generation of active templates

In the experiments described by Worcel and coworkers (1,17) ATP was found to be required for the generation of torsionally unconstrained supercoils and transcriptionally active 5S gene templates. In the experiment of Figure 2 we also monitored the effect of added ATP (3 mM) on both DNA topology and transcriptional activity. Although some stimulation of transcription is achieved with additional ATP, no pronounced effect on supercoiling is observed. This experiment, however, is complicated by the endogenous ATP found in the S-150 extract. We have found, however, that some S-150 extracts are absolutely dependent on the addition of exogenous ATP in a preincubation step for the generation of active templates (Figure 5A). Preincubation in the absence of ATP renders the template largely inactive (lane 4). The other ribonucleoside triphosphates will substitute for ATP, but the nonhydrolyzable ATP analogue AMP-PNP will not (lane 5). For those extracts which are not dependent on exogenous ATP during the preincubation step, we depleted the extract of endogenous ATP with hexokinase and glucose and then tested the effect of adding back ATP during the preincubation step. Figure 5B shows that depletion markedly reduces the level of transcription (compare lane 1 with lanes 2 and 3) and that ATP can reverse the effect of ATP depletion (lane 6). As before, AMP-PNP will not substitute for ATP in this experiment (lanes 4 and 5). Different results have been obtained with the HeLa S-100 extract (30, see discussion). We find that AMP-PNP is not inhibitory to polymerase III transcription in the presence of the other nucleotides; in fact, AMP-PNP can be utilized by eukaryotic RNA polymerase III for transcription (30). We have not determined whether the S-150 possesses the enzymatic activities needed to generate ATP from the other ribonucleoside triphosphates. Deoxynucleotides do not substitute in the preincubation step for their ribonucleotide counterparts (not shown).

As it is conceivable that the ATP-dependant preincubation step could reflect some enzymatic reaction involving only the polymerase III transcription factors or polymerase itself, we examined the requirement for DNA during the preincubation step. We find that

Figure 5. Nucleotide requirement in the preincubation step. Panel A. 30 μ l aliquots of an S-150 extract were preincubated for 3 h with 500 ng 5S DNA and either 1.6 mM ATP (lane 1), CTP (lane 2), UTP (lane 3), no nucleotide (lane 4) or AMP-PNP (lane 5). Preincubations also contained 6 mM $MgCl_2$. Products of transcription in a subsequent 2 h reaction with all four nucleotides and labeled GTP are shown. Panel B. 30 μ l aliquots of an S-150 extract were depleted of ATP by preincubation with hexokinase and dextrose (1 mM) for 1 h. Lane 1, no treatment. Lanes 2 and 4 contained 0.01 units of hexokinase and lanes 3, 5 and 6 contained 0.02 units. After the following additions were made, the extract and 500 ng 5S DNA were preincubated for 3 h prior to the initiation of transcription: lanes 1, 2 and 3, no addition; lanes 4 and 5, 1.6 mM AMP-PNP; lane 6, 1.6 mM ATP.

DNA must indeed be present during this step and that extracts preincubated with ATP in the absence of DNA do not support high levels of transcription (data not shown).

Role of topoisomerases in the generation of active templates

The well characterized eukaryotic DNA topoisomerases I and II can be inhibited *in vitro* by the antitumor drugs camptothecin and VM-26, respectively (34,35). We have added these drugs to the S-150 either alone or in combination in sufficient quantity to inhibit their target enzymes. All detectable topoisomerase activity in the S-150 is inhibited by these drugs in combination (data not shown; 21). The effect of these drugs on 5S

Figure 6. Phosphorylation of a 5S DNA binding protein during preincubation in the S-150. 30 μ l aliquots of the S-150 were incubated for 3 h at 22°C with no DNA (lane 1), 500 ng pUC19 DNA (lane 2), or 500 ng pXls11 DNA (lanes 3–5). The reactions also contained 10 μ Ci of ^{32}P -ATP, 6mM $MgCl_2$ and 1mM unlabeled ATP. $MgCl_2$ was omitted from the reaction of lane 3 and unlabeled ATP was omitted from the reaction of lane 5. After incubation the samples were chromatographed on 1 ml columns of Sepharose 6B-CL. Void volume fractions (containing DNA) were collected and subjected to electrophoresis on SDS polyacrylamide gels. The gel was stained with coomassie blue to detect total protein and subjected to autoradiography. The autoradiogram is shown. M denotes positions of molecular weight markers.

Figure 7. Binding of ^{32}P -labeled proteins to a 5S DNA restriction fragment as detected by a gel mobility shift assay. A 248bp fragment containing the 5S RNA gene and 49bp of 5' flanking sequence and 69bp of 3' flanking sequence was subjected to electrophoresis in a nondenaturing gel in the presence of S-150 proteins. In addition to the labeled DNA, 1 μg of plasmid DNA digested with Bam HI to liberate the 248 bp fragment was included in the incubations. Panel A shows the autoradiogram of ^{32}P -labeled DNA either in absence (lane 1) or presence (lane 2) of 30 μl of S150. Panel B shows a Western blot of a similar nondenaturing gel as that shown in Panel A, lane 2, but probed with a polyclonal anti-TFIIIA antibody. The major band represents the 7S RNP containing 5S RNA and TFIIIA. Panel C shows the distribution of ^{32}P -labeled S-150 proteins in a nondenaturing gel as detected by autoradiography. S150 proteins were phosphorylated as described in the legend to Figure 6. Panel D shows the autoradiogram of a second dimension SDS gel of the ^{32}P -labeled S150 proteins run in the nondenaturing gel of Panel C. The relative migration of molecular weight markers (in kDa) are indicated.

transcription in the S-150 was monitored in the same fashion as done previously for the HeLa S-100 (31). We find that neither drug alone nor the combination are inhibitory to 5S transcription in the S-150 (data not shown). Similar results have been obtained with the HeLa extract (31) and with *Xenopus* egg extracts (21).

Phosphorylation of DNA binding proteins during preincubation in the S-150

As the requirement for ATP in the generation of active 5S gene templates could reflect protein phosphorylation, we examined the phosphorylation of S-150 proteins during preincubation in the S-150. Since the preincubation step requires 5S gene-containing plasmids, we looked for DNA-dependant phosphorylation. To accomplish this, we incubated equivalent 30 μl aliquots of the S-150 with either no DNA, pUC19 vector DNA or pXls11 5S DNA and labeled and unlabeled ATP. We next separated DNA bound proteins (and large protein complexes) from free proteins by chromatography on Sepharose 6B and analyzed the proteins in the DNA-containing fractions by SDS-gel electrophoresis and autoradiography (Figure 6). We find that one high molecular weight protein species is

labeled with ^{32}P in a 5S DNA dependent preincubation (lane 4, arrow). The approximate molecular mass of this polypeptide is 200 kD. This protein is only weakly labeled in the absence of DNA (lane 1) or in the presence of pUC19 DNA (lane 2). Labeling of this protein also requires MgCl_2 and unlabeled ATP (lanes 3 and 5). Phosphorylation of this protein is stimulated 3 to 5-fold by 5S gene plasmid DNA over pUC19 DNA or no DNA. A polypeptide of 22 kD is also phosphorylated to a greater extent in the presence of 5S DNA.

We employed the gel mobility shift assay (36,37) to examine whether the 22 and 200 kD phosphoproteins bind to the 5S gene region of the plasmid. A restriction fragment of 248 bp bearing the somatic-type 5S gene and 49 bp of 5' and 69 bp of 3' flanking sequence was subjected to nondenaturing gel electrophoresis after incubation in the absence (Figure 7, panel A, lane 1) or presence of the S-150 (lane 2). The 5S gene fragment migrates as a large protein-DNA complex after incubation in the S-150. This complex has a much lower electrophoretic mobility than TFIIIA-DNA complexes (not shown, but see 10,19) and presumably contains proteins in addition to TFIIIA. A western blot of an equivalent gel lane was probed with an antiserum to TFIIIA to monitor the distribution of this protein in the nondenaturing gel (panel B). Most of the TFIIIA is detected as 7S RNPs (TFIIIA-5S RNA complexes) while a significant fraction (about 15%) migrates at a position coincident with the 5S DNA restriction fragment. Phosphorylated proteins were examined by autoradiography of the nondenaturing gel after inclusion of $\gamma^{32}\text{P}$ -ATP along with the 5S DNA fragment and the S-150 in the preincubation (panel C). A diffuse doublet of radioactive material is observed, with the upper region of the doublet coincident with the position of the 5S DNA fragment. A second dimension of SDS gel electrophoresis followed by autoradiography displays the molecular weights of the ^{32}P -polypeptides in the first dimension nondenaturing gel (panel D). From this analysis the 22 kD phosphoprotein does not appear to be specifically associated with the 5S restriction fragment; this polypeptide migrates in the nondenaturing gel behind the 5S fragment. On the other hand, the 200 kD polypeptide is found in the region of the first dimension gel containing the 5S DNA fragment. A significant fraction of the 200 kD polypeptide migrates ahead of the 5S DNA suggesting that not all of this protein is complexed to the DNA. Nonetheless, these data suggest that at least a fraction of the 200 kD polypeptide is complexed with the 5S gene fragment and that this protein may be an important component of the transcription complex. Further study will be needed to provide evidence for or against the involvement of this protein in 5S gene transcription.

DISCUSSION

The results presented above show that the optimum conditions for DNA supercoiling in the S-150 differ dramatically from the optimum conditions for the generation of a transcriptionally competent 5S gene template. Numerous studies have shown that supercoiling in oocyte and egg extracts is a consequence of nucleosome assembly (1,13,32,38). In agreement, we find a nucleosome-like DNase I footprint on the 5S gene incubated in the S-150 under supercoiling conditions (Figure 4). We find no evidence in the footprint for the association of TFIIIA with these DNA molecules; rather, the footprint resembles that of a positioned nucleosome reconstituted on the same *X. laevis* 5S gene with purified histones (20). Careful inspection of the TFIIIA and nucleosome footprints on the 5S genes reveals common sites of close contact of the DNA with both TFIIIA and the nucleosomal histones suggesting that both proteins recognize and bind the same helical

face of 5S DNA (19,20). This provides a molecular explanation for why a nucleosome excludes TFIIIA binding (Figure 4 and ref. 20). In contrast to the results obtained under optimal supercoiling conditions, optimal transcription conditions in the S-150 lead to a TFIIIA footprint on the same 5S gene (23,24) and plasmid molecules bearing few nucleosomes as evidenced by a low superhelical density (Figure 1A).

Thus our present results are in agreement with previous studies for the 5S genes (13,14,21) and the adenovirus major late promoter (38–41). In the case of 5S genes, TFIIIA binding must precede nucleosome formation to yield an active template and nucleosome assembly precludes transcription factor binding. Similarly, transcription complexes must be formed on the major late promoter prior to nucleosome assembly for promoter activity. Plasmids containing two-thirds of the *in vivo* nucleosome density were found to be inactive in transcription when subsequently supplied with the necessary factors (39). Workman and Roeder (38) demonstrated that the polymerase II transcription factor required to maintain promoter function during nucleosome assembly was the TATA box binding factor TFIID. Although nucleosomes may block the binding of activating factors, nucleosomes are not a barrier to polymerase passage (40). Therefore, nucleosome positioning on promoter elements could provide a level of control of gene expression in addition to the availability of specific transacting factors.

In studies with partially purified transcription factors from HeLa cells, Roeder and his colleagues showed that the rate-limiting step in 5S transcription is the magnesium and ATP-dependent association of TFIIB with the TFIIIA-TFIIC-DNA complex (5). Further studies indicated that TFIIB binding could be in the absence of ATP and subsequent nucleotide interaction was required for the generation of a primed template (30). Interestingly, any nucleoside triphosphate, including the nonhydrolyzable ATP analogue AMP-PNP, could satisfy the nucleotide requirement. Our results for the *Xenopus* S-150 differ in that AMP-PNP will not substitute for hydrolyzable nucleotides (Figure 5); however, this nucleotide is not inhibitory to transcription. In fact, Bieker and Roeder have shown that human RNA polymerase III can utilize AMP-PNP as the sole adenosine-containing ribonucleotide. Thus hydrolysis of the alpha-beta bond for RNA synthesis is not blocked by the nonhydrolyzable beta-gamma bond. Our results suggest that hydrolysis of a beta-gamma bond is required, however, for the generation of the active transcription complex, suggesting a difference between the *Xenopus* and HeLa systems.

Results with inhibitors of DNA topoisomerases I and II indicate that these enzymes are not involved in transcription complex formation. Similarly, DNA supercoiling does not appear to correlate with transcriptional activity as previously suggested (17). Finally, we examined the phosphorylation of DNA binding proteins during the preincubation step in the S-150. Interestingly, we find that a 200 kD protein is phosphorylated in a 5S gene-specific fashion. This protein is only weakly phosphorylated in the absence of 5S gene containing plasmids. The results of combined nondenaturing gel electrophoresis and second dimension SDS gel electrophoresis suggest that this protein might be a component of the transcription complex. That this protein might be a subunit of TFIIC is suggested both by the high molecular weight of TFIIC from yeast and mammalian sources (42) and the activation and phosphorylation of HeLa TFIIC upon adenovirus infection (43); however, further investigation will be needed to provide a direct correlation between this protein and 5S gene transcription. It is conceivable that the nucleotide requirement for the generation of transcription complexes may reflect DNA-dependent protein phosphorylation and specifically the phosphorylation of this 200 kD polypeptide.

ACKNOWLEDGEMENTS

We thank Drs. D.D. Brown and A. Wolffe for gifts of plasmids and Mary Keeter for preparation of the manuscript. This work was supported by grants from the National Institutes of Health (GM26453) and the American Cancer Society (FRA 292) and is publication number 5518MB from the Research Institute of Scripps Clinic.

REFERENCES

1. Glikin, G.C., Ruberti, I., Worcel, A. (1984) *Cell* 37, 33–41.
2. Korn, L.J. (1982) *Nature* 295, 101–105.
3. Brown, D.D. (1984) *Cell* 37, 359–365.
4. Shastry, B.S., Ng, S.-Y., Roeder, R.G. (1982) *J. Biol. Chem.* 257, 12979–12986.
5. Bieker, J.J., Martin, P.L., Roeder R.G. (1985) *Cell* 40, 119–127.
6. Engelke, D.R., Ng, S.-Y., Shastry, B.S., Roeder, R.G. (1980) *Cell* 19, 717–728.
7. Sakonju, S., Brown, D.D. (1982) *Cell* 31, 395–405.
8. Ginsberg, A.M., King, B.O., Roeder, R.G. (1984) *Cell* 39, 479–489.
9. Tso, J.Y., Van Den Berg, D.J., Korn, L.J. (1986) *Nucl. Acids Res.* 14, 2187–2200.
10. Smith, D.R., Jackson, I.J., Brown, D.D. (1984) *Cell* 37, 645½652.
11. Miller, J., McLachlan, A.D., Klug, A. (1985) *EMBO J.* 4, 1609–1614.
12. Setzer, D.R., Brown, D.D. (1985) *J. Biol. Chem.* 260, 2483–2492.
13. Gottesfeld, J., Bloomer, L.S. (1982) *Cell* 28, 781–791.
14. Bogenhagen, D.F., Wormington, W.M., Brown D.D. (1982) *Cell* 28, 413–421.
15. Schlissel, M.S., Brown, D.D. (1984) *Cell* 37, 903–913.
16. Ryoji, M., Worcel, A. (1984) *Cell* 37, 21–32.
17. Kmiec, E.B., Worcel, A. (1985) *Cell* 41, 945–953.
18. Kmiec, E.B., Ryoji, M., Worcel, A. (1986) *Proc. Natl. Acad. Sci. USA* 83, 1305–1309.
19. Rhodes, D. (1985) *EMBO J.* 4, 3473–3482.
20. Gottesfeld, J.M. (1987) *Mol. Cell. Biol.* 7, 1612–1622.
21. Wolffe, A.P., Andrews, M.T., Crawford, E., Losa, R., Brown, D.D. (1987) *Cell* 49, 301–303.
22. Shimamura, A., Trementhick, D., Worcel, A. (1988) *Mol. Cell. Biol.* 8, 4257–4269.
23. Peck, L.J., Millstein, L., Eversole-Cire, P., Gottesfeld, J.M., Varshavsky, A. (1987) *Mol. Cel. Biol.* 7, 3503–3510.
24. Millstein, L., Eversole-Cire, P., Blanco, J., Gottesfeld, J.M. (1987) *J. Biol. Chem.* 262, 17100–17110.
25. Wolffe, A.P., Brown, D.D. (1986) *Cell* 47, 217–227.
26. Wolffe, A.P., Brown, D.D. (1987) *Cell* 51, 733–740.
27. Blanco, J., Millstein, L., Razik, M.A., Dilworth, S., Cote, C., Gottesfeld, J.M. (1988) Submitted.
28. Korn, L.J., Gurdon, J.B. (1981) *Nature* 289, 461–465.
29. Birkenmeier, E.H., Brown, D.D., Jordan, E. (1978) *Cell* 15, 1077–1086.
30. Bieker, J.J., Roeder, R.G. (1986) *J. Biol. Chem.* 261, 9732–9738.
31. Gottesfeld, J.M. (1986). *Nucl. Acids Res.* 14, 2075–2088.
32. Laskey, R.A., Mills, A.D., Morris, N.R. (1977) *Cell* 10, 237–243.
33. Simpson, R.T., Thoma, F., Brubaker, J.M. (1985) *Cell* 42, 799–808.
34. Hsiang, Y.-H., Hertzberg, R., Hecht, S., Liu, L.F. (1985) *J. Biol. Chem.* 260, 14873–14878.
35. Chen, G.L., Yang, L., Rowe, T.C., Halligan, B.D., Tewey, K.M., Liu, L.F. (1984) *J. Biol. Chem.* 259, 13560–13566.
36. Fried, M., Crothers, D. (1981) *Nucl. Acids Res.* 9, 6505–6525.
37. Garner, M., Revzin, A. (1981) *Nucl. Acids Res.* 9, 3047–3060.
38. Workman, J.L., Roeder, R.G. (1987) *Cell* 51, 613–622.
39. Knezetic, J.A., Luse, D.S. (1986) *Cell* 45, 95–104.
40. Lorch, Y., LaPointe, J.W., Kornberg, R.D. (1987) *Cell* 49, 203–210.
41. Matsui, T. (1987) *Mol. Cell. Biol.* 7, 1401–1408.
42. Geiduschek, E.P., Tocchini-Valentini, G. (1988). *Ann. Rev. Biochem.* in press.
43. Hoeffler, W.K., Kovelman, R., Roeder, R.G. (1988) *Cell* 53, 907–920.

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