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RNA Polymerase III Transcription in Synthetic Nuclei Assembled In Vitro from Defined DNA Templates

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Although much is known of the basic control of transcription, little is understood of the way in which the structural organization of the nucleus affects transcription. Synthetic nuclei, assembled de novo in extracts of *Xenopus* eggs, would be predicted to have a large potential for approaching the role of nuclear structure in RNA biogenesis. Synthetic nuclei provide a system in which the genetic content of the nuclei, as well as the structural and enzymatic proteins within the nuclei, can be manipulated. In this study, we have begun to examine transcription in such nuclei by using the most simple of templates, RNA polymerase III (pol III)-transcribed genes. DNA encoding tRNA or 5S genes was added to an assembly extract, and nuclei were formed entirely from the pol III templates. Conditions which allowed nuclear assembly and pol III transcription to take place efficiently and simultaneously in the assembly extract were found. To examine whether pol III transcription could initiate within synthetic nuclei, or instead was inhibited in nuclei and initiated only on rare unincorporated templates, we identified transcriptional inhibitors that were excluded from nuclei. We found that these inhibitors, heparin and dextran sulfate, blocked pol III transcription in the absence of assembly but did not do so following nuclear assembly. At the concentrations used, the inhibitors had no deleterious effect on nuclear structure itself or on nuclear import. We conclude that pol III transcription is active in synthetic nuclei, and this conclusion is further strengthened by the finding that pol III transcripts could be coisolated with synthetic nuclei. The rapid and direct transcriptional analysis possible with pol III templates, coupled with the simple experimental criteria developed in this study for distinguishing between nuclear and nonnuclear transcription, should now allow a molecular analysis of the effect of nuclear structure on transcriptional and posttranscriptional control.

In the study of organelles, an in vitro system which allows reconstitution of an organelle from its component parts is greatly valued. For the nucleus, such a reconstitution system has been developed and uses as its basis an extract of *Xenopus laevis* eggs. Each *Xenopus* egg contains a stockpile of all nuclear components necessary to assemble up to 4,000 somatic nuclei, stored for the rapid divisions which take place in the early embryo (for reviews, see references 5 and 65). Egg extracts thus provide a concentrated source of disassembled nuclear components. When chromatin substrates, derived from demembranated sperm, are added to a crude extract of *Xenopus* eggs, they undergo an ordered series of events which results in the formation of nuclei. These reconstituted nuclei are morphologically identical to normal nuclei in many structural aspects; they are enclosed by inner and outer nuclear membranes and contain nuclear pores and an underlying nuclear lamina (12, 57, 58, 70, 72, 78). Reconstituted nuclei carry out a number of hallmark functions of normal nuclei, including semiconservative DNA replication and nuclear protein import (12, 62, 63, 70). Moreover, when the mitotic kinase cyclin/cdc2 is added to such sperm chromatin nuclei, mitotic disassembly of the nuclei rapidly ensues (56, 59, 71).

Protein-free DNA itself is a template for nuclear formation. When bacteriophage λ DNA, or indeed double-stranded DNA from any source, is injected into *Xenopus* eggs, synthetic nuclei assemble within the egg cytosol in large numbers (28). An identical assembly of synthetic nuclei occurs when double-stranded DNA is added to cell extracts of *Xenopus* eggs (12, 14, 15, 33, 50, 62, 64, 66–68). Both in vitro and in vivo, the assembly process involves nucleosome formation, compaction into higher-order chromatin intermediates, binding of nuclear membrane vesicles, and fusion of the vesicles into double nuclear membranes containing nuclear pores (12, 62, 64, 68). The synthetic nuclei are functional for nuclear protein import (33, 62, 64, 66). Moreover, once the double-stranded DNA templates become incorporated into nuclei, semiconservative replication occurs, originating at discrete subnuclear locations which resemble the replication foci seen in eukaryotic nuclei (14). These studies indicated that eukaryotic DNA sequences are not necessary for the formation of much of nuclear structure and, in addition, that nuclear structure can be experimentally manipulated in vitro.

Immunodepletion of nuclear assembly extracts is an additional powerful tool for the molecular dissection of the nucleus. Specific proteins can be depleted from the extract prior to assembly, and an altered nucleus can then be formed. The effect of depletion on both nuclear biogenesis and function can be tested. Immunodepletion of single nuclear pore proteins from extracts has allowed the assembly of nuclei with modified pores (25, 26, 76). Similar depletion studies have been used to elucidate such fundamental nuclear processes as DNA replication, mitotic chromosome organization, cell cycle control, and nuclear envelope assembly (1, 17, 22, 35, 72, 92).

One of the most important activities of the eukaryotic nucleus is the transcription of RNA. There is little understanding, however, of the way in which the myriad layers of structural organization within the nucleus affect transcription. The approach described above, in which nuclear architecture is reconstituted and manipulated, has been applied to only a limited extent toward the analysis of RNA transcription. Instead, transcription has been studied primarily with simple in vitro

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systems which combine naked DNA templates with isolated transcription factors or with in vivo systems involving transfected tissue culture cells or transgenic animals. While a large amount of information has been gained through these studies, differences between the in vitro and in vivo systems indicate that essential regulatory features are not recapitulated in the simple systems (for examples, see references 4 and 51 and references therein). This finding has led investigators to assess the contribution of chromatin structure to transcriptional control. The importance of chromatin in regulatory mechanisms has now been established, but this in turn has raised other questions (see references 4, 6, 9, 43, 51, 54, 74, 84, and 90 and references therein). For example, the dynamic nature of chromatin points toward an additional level of transcriptional regulation that takes place during DNA replication, when DNA is transiently more accessible to transcription factors. This level of control has been difficult to approach in the more basic systems, since the initiation of replication on double-stranded templates occurs accurately and efficiently only when DNA is enclosed in a nucleus (12, 53, 68; see Discussion). Further questions have been raised by recent findings with fluorescence and electron microscopy indicating that transcription and replication in reality take place in particular nuclear subdomains (13, 34, 36, 37, 39, 40, 53, 60, 81, 85). In addition, when specific transcripts were monitored with in situ hybridization, the results suggested that the biogenesis of mRNA occurs, at least in some instances, along anchored paths or tracks (38, 91). These higher-order patterns of template organization within the nucleus may exert important regulatory influences on transcription, yet an examination of the role of higher-order structure has not been accessible to biochemical analysis.

Synthetic nuclei provide a system wherein both the transcription template and nuclear structure can be manipulated. In such a system, the specific DNA template used to assemble nuclei can be varied. Distal regulatory regions, such as enhancers or matrix attachment regions (MARs), can be included in *cis* with a template, and the effect on transcription can be assessed. The structure of the synthetic nuclei can also be altered. Proteins of interest, such as MAR-binding proteins or putative nuclear matrix proteins, can be immunodepleted from an extract, nuclei can be formed in their absence, and the effect on template transcription can be assayed.

Preliminary reports point toward the potential of *Xenopus* egg extracts for examining higher levels of transcriptional control. Mitotic and interphase cytosols have been used in combination with cloned genes to elucidate the mechanism of transcriptional repression observed at mitosis, although this level of control does not require the presence of nuclear structure (30–32). Nuclei assembled around sperm chromatin, a complex genomic template, have been used to demonstrate that the timing of DNA replication has no effect on the differential expression of oocyte and somatic 5S genes (87). Thus, this type of control apparently does not require this particular nuclear function. In another study, *Xenopus* egg extracts have been combined with nuclear extracts of chicken erythrocytes containing erythroid cell-specific polymerase II (pol II) transcription factors to look at chromatin remodeling and transcription of a cloned β-globin gene. Initial observations suggest that nuclei form and that replication allows increased accessibility of the β-globin regulatory region (8; see Discussion). Although interesting, this is a very complex system which, though appropriate for analysis of aspects of globin transcription, is not amenable to a basic biochemical study of nuclear structure.

To specifically address the role of nuclear structure on transcription, one would ideally start with a simple template and a basic assembly extract. A simple pol III-transcribed gene, such as a tRNA gene, would be predicted to be the most basic of genes to examine in this context. Although egg cytosol has been used extensively to study pol III transcription, a pol III template has never been assembled into nuclei and tested for the ability to direct pol III transcription. In this study, we assembled nuclei in vitro by using a defined tRNA template. We established conditions in which RNA pol III is active in the presence of assembled nuclei. We next evaluated whether the transcripts produced originate from nuclear templates or from templates which had escaped incorporation into nuclei. We found that the majority, if not all, of the transcripts observed were directed by templates within the reconstituted nuclei and that newly made transcripts could be coisolated with synthetic nuclei. Thus, synthetic nuclei show vigorous RNA pol III transcription and do so without any exogenously added transcription factors, making this a simple system for studying nuclear transcription.

MATERIALS AND METHODS

Plasmids and reagents. phH2DΔ3-5 (referred to as pΔ3) was a kind gift from M. Zasloff (2). pPC-1 (referred to as pPC) and pXls11 (referred to as pXls) were originally obtained from P. Geiduschek and J. Gottesfeld, respectively (45, 75). TFIIIA, partially purified from *Xenopus* oocytes (see reference 79; heparin chromatography was substituted for the Biorex-70 step), was generously provided by J. Gottesfeld. Dextran sulfate, heparin, *N*-ethylmaleimide (NEM), Ficoll (type 400), and spermidine were purchased from Sigma. α-Amanitin was purchased from Boehringer Mannheim. RNasin was purchased from Promega. The fluorescent membrane dye 3,3′-dihexyloxacarbocyanine iodide (DHCC) was purchased from Kodak.

Preparation of fractionated egg extracts. Eggs were dejellied in 2% cysteine (pH 7.8). The cysteine solution was then removed by washing with MMR (100 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 0.1 mM EDTA, 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.8]), and the eggs were transferred into MMR containing 4% Ficoll, cycloheximide (50 μg/ml), and calcium ionophore A23187 (0.2 μg/ml; Calbiochem). After 5 min, the eggs were rinsed with 0.25× MMR containing 3% Ficoll and cycloheximide (50 μg/ml) and then equilibrated into egg lysis buffer (ELB; 250 mM sucrose, 2.5 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol [DTT], 50 μg of cycloheximide per ml, 10 mM HEPES [pH 7.6]). Egg extracts were prepared essentially as described by Hartl et al. (33). After a low-speed centrifugation separation, the final cytosolic fraction was obtained by high-speed ultracentrifugation (250,000 × *g*) for 90 min and then clarified with a second spin (250,000 × *g*) for 30 min. The final membrane pellet was resuspended by gently pipetting and was used as a 15× stock. Glycogen was purified from the gelatinous pellet obtained after the first high-speed spin as described by Kennedy et al. (33, 46). Commercially available glycogen (oyster glycogen; Calbiochem) can also be used with no further purification (33).

DNA templates. Bacteriophage lambda DNA was prepared from CsCl-banded phage by using standard protocols. Ligated plasmid DNA was produced as follows. CsCl-banded plasmid template DNA was linearized with an appropriate restriction enzyme (*Hin*dIII for pPC and pUC9, *Sal*I for p2D and pΔ3, and *Bam*HI for pXls). A second filler plasmid that did not contain a transcription template (Bluescript; Stratagene) was digested in parallel reactions with the same enzymes. Pairs of plasmids cut with corresponding enzymes were ligated at a high concentration (0.8 to 1 mg/ml) at a template/filler ratio of 25:1. After overnight ligation at 4°C, the ligase was heat inactivated (15 min at 65°C), and the reaction mixture was diluted ~10-fold into a mixture containing *Not*I, which recognizes a unique site found only in the filler plasmid (resulting in an average template size of ~75 to 125 kb). In this way, long linear templates were generated, but the total length was somewhat restricted. This offered a compromise between length of the template, which was observed to increase nuclear assembly efficiency greatly, and transcriptional activity, which was observed to be inversely proportional to template length. Transcription was found to decrease ~10-fold (data not shown) when the template/filler ratio was changed from 5:1 (~15 kb) to 50:1 (~150 kb).

Nuclear assembly and transcription assays. Sperm chromatin nuclei (~1,000/μl) were assembled in the presence of cytosol (25 μl), membranes (2 μl), and an ATP-regenerating system (1 μl) with final concentrations of 20 mM phosphocreatine, 2 mM ATP, and 50 μg of creatine kinase per ml (65, 80). Following a 60-min incubation at room temperature, the reaction mixture was supplemented with 30 mM KCl, 4 mM DTT, and 2 mM $MgCl_2$ for optimal transcription. Transcripts were then labeled for 60 min by the inclusion of 10 μCi of [α-^{32}P]GTP and prepared for analysis as described below.

Nuclear assembly around naked DNA templates (250 ng of λ or ligated plasmid DNA) was carried out as described by Hartl et al. (33) by initially

incubating the DNA with 25 μl of cytosol, 600 μg of glycogen, and 1 μl of ATP-regenerating mixture. When 5S transcription was monitored, the ligated DNA template was preincubated with TFIIIA (~100 ng/250 ng of plasmid) in buffer containing 100 mM KCl, 20 mM HEPES (pH 7.6), 10% glycerol, 0.1 mM EDTA, 6 mM $MgCl_2$, and 25 μM zinc acetate (~42 ng of DNA per μl). Following a 10-min incubation, pXls and TFIIIA were added to a nuclear assembly reaction mixture as described above. After the formation of chromatin intermediates in the presence of glycogen, spermidine (1 mM, final concentration) and the vesicular membrane fraction (2 μl) were added. Transcriptional inhibitors, if used, were added 90 min after the addition of membranes at the concentrations indicated in the figure legends. For reactions mixtures in which membranes or inhibitors were not included, the appropriate volume adjustment was made with ELB or water, respectively. The salt concentration was also adjusted, as indicated for individual experiments in the figure legends, and the reaction mixtures were supplemented with 4 mM DTT 90 min after membrane addition.

An aliquot of this mixture (18 to 25 μl) was then taken, and 10 μCi of [α-^{32}P]GTP (ICN) was added in a volume of 2 μl containing 5 to 10 U of RNasin and ~1 μl of ELB. Rhodamine-labeled human serum albumin coupled to the T-antigen nuclear localization sequence peptide (6 to 12 μg/ml) was added to the remainder of the nuclear assembly reaction mixture to assess the formation of functional nuclei by their ability to import (63). After 30 to 90 min of [α-^{32}P]GTP incorporation, the reaction was stopped by the addition of 200 μl of buffer containing 0.5 M NaCl, 25 mM EDTA, 0.5% sodium dodecyl sulfate, and 10 mM Tris (pH 7.6). The samples were then phenol extracted, ethanol precipitated, and electrophoresed on a denaturing acrylamide gel (8 M urea, 6% acrylamide). An aliquot (4 μl) of the parallel reaction mixture containing transport substrate was examined by fluorescence microscopy, following fixation with 1 μl of 11% formaldehyde in 250 mM sucrose–10 mM HEPES (pH 7.5) plus the membrane stain DHCC and the fluorescent DNA dye Hoechst H33258 (Calbiochem) at 10 μg/ml each.

Heparin and dextran sulfate were titrated to find concentrations at which nuclear structure was unaffected, as assessed by a visual monitoring of nuclear import, nuclear size, and the presence of an intact nuclear envelope. Once this concentration was determined, the ability of the inhibitor to block transcription was assessed. Heparin at high concentrations (200 μg/ml) has been reported to block Ca^{2+} release from inositol trisphosphate receptors and to consequently inhibit nuclear vesicle fusion (82). However, this was unlikely to occur under the conditions used here, both because the concentration of heparin used was ~10-fold less (20 μg/ml) and because heparin was added only after formation of nuclear envelopes had occurred.

When testing the effect of dextran sulfate on transcripts encoded by nuclear or free chromatin templates in mixing experiments, we first set up reactions in parallel, with membrane vesicles added only to the nuclear assembly reaction. The two types of reactions—in which one template was assembled into nuclei and the other template was nonnuclear—were combined after nuclear formation had taken place, i.e., 90 min after membrane addition. Dextran sulfate was then added, and after 5 min, transcripts were labeled with [α-^{32}P]GTP for 30 min.

NEM treatment of membranes. NEM (30 mM) was diluted 1:10 into an aliquot of membrane vesicles derived as described above. Following incubation at room temperature for 4 min, 60 mM DTT was added for another 4-min incubation, also at a 1:10 dilution. The control membranes were treated in the opposite order.

Sucrose step gradient. Nuclei were assembled as described above. Label, [α-^{32}P]GTP, was added, and transcription was allowed to continue for 45 min. When both nuclear and nonnuclear transcripts were going to be assayed, the nonnuclear templates were added to the reaction mixture immediately prior to the addition of [α-^{32}P]GTP. Following 45 min of radiolabel incorporation, samples were placed on ice. An aliquot of vesicular membranes was added to the sample containing no nuclei to control for any nonspecific effects of the membrane components during the spin-down procedure. After 10 min on ice, the samples were diluted with 10 volumes of ELB containing 1.4 M sucrose. An aliquot of these diluted reaction mixtures (100 μl) was layered onto a sucrose step gradient composed of a 20-μl cushion of 2.0 M sucrose–ELB, 80 μl of 1.7 M sucrose–ELB, and 50 μl of 1.5 M sucrose–ELB in a polyallomer tube (catalog no. 342630; Beckman). Using adaptors (catalog no. 358614; Beckman), the gradients were centrifuged 20 min at 40,000 rpm (35,000 × *g*) in a TLS-55 rotor (Beckman). The material in the gradients was recovered in two 125-μl aliquots, and following phenol extraction and ethanol precipitation, the transcripts were analyzed by gel electrophoresis.

RESULTS

RNA pol III transcription occurring under the conditions of nuclear assembly is resistant to inhibition by heparin or dextran sulfate. To develop an in vitro system for analyzing nuclear transcription, crude *Xenopus* egg extracts were prepared and fractionated by high-speed centrifugation into cytosolic, membrane vesicular, and pellet fractions. As previously described, all three fractions are required for efficient nuclear formation around naked DNA templates (33). The cytosol serves as a source of histones and nonchromosomal nuclear proteins, while the vesicular fraction contains nuclear-specific vesicles in a disassembled state (68). The active component of the pellet fraction proves to be glycogen, which promotes packaging of the DNA into a higher-order chromatin intermediate needed for efficient nuclear formation (33). To generate nuclei containing pol III templates, protein-free DNA containing a tRNA gene was incubated in *Xenopus* egg cytosol supplemented with glycogen (Fig. 1A). After 1 h, either buffer or an aliquot of the vesicular membrane fraction was added. To ensure complete assembly of nuclei in the membrane-supplemented reaction mixture, samples were incubated for a further 90 min prior to the addition of [α-^{32}P]GTP. Incorporation of radiolabel was then allowed to occur for 30 to 90 min to examine whether pol III transcription takes place under conditions of nuclear assembly. The RNA was prepared from

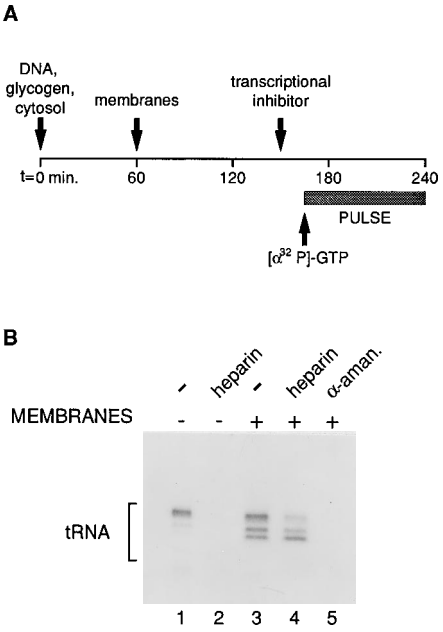

FIG. 1. The conditions of nuclear formation prevent the decrease in transcription caused by heparin but not α-amanitin. (A) The standard experimental design for nuclear assembly of naked DNA templates, followed by the measurement of RNA pol III transcription, is outlined. For a typical experiment, DNA and glycogen are first incubated together in the high-speed cytosolic fraction of *Xenopus* eggs. After 1 h, an aliquot of the vesicular membrane fraction is added or, in parallel reactions in which nuclei were not formed, buffer is added instead. After another 90 min during which time the nuclear assembly process is completed, transcriptional inhibitors are mixed into specific reactions, followed by the addition of 10 μCi of [α-^{32}P]GTP to each sample. The pulse of incorporation is allowed to proceed for 30 to 90 min, as indicated, after which time the reaction is processed for analysis on polyacrylamide gels. In cases in which the nuclei are monitored visually as well, transport substrate is added to an aliquot of the reaction mixture taken immediately prior to the addition of radioactive GTP. (B) An experiment was performed as described above, using a bacteriophage λ template containing the yeast $tRNA_3^{Leu}$ gene (33). Transcription was monitored in the absence (lanes 1 and 2) and presence (lanes 3 to 5) of the vesicular membrane fraction and consequent nuclear formation. Transcripts were pulsed with [α-^{32}P]GTP for 90 min. The primary transcript detected is believed to correspond to the unprocessed product of this gene, while the faster-migrating products are likely to be transcripts that have undergone various amounts of 5′ and 3′ processing. In these reactions, the buffer contained 80 mM KCl and 2.5 mM $MgCl_2$. Transcription of yeast $tRNA_3^{Leu}$ was inhibited by heparin (20 μg/ml) when no membranes were present (lane 2). When membranes were present and nuclear formation took place, transcription was much less affected by the addition of heparin (lane 4). In contrast, α-amanitin (α-aman.; 100 μg/ml) blocked tRNA transcription even under the conditions of nuclear formation (lane 5).

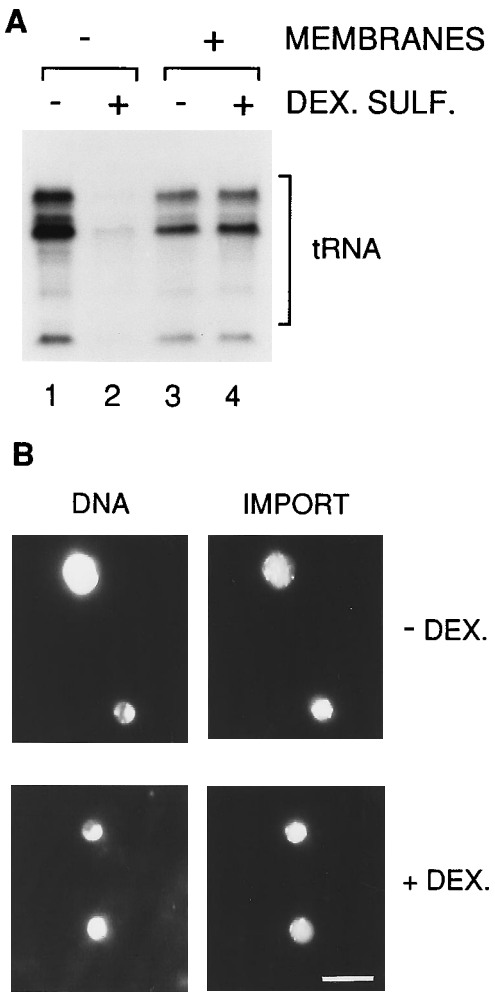

FIG. 2. Nuclear formation prevents the inhibition of transcription by dextran sulfate. (A) Plasmid DNA (pPC) was incubated in the presence or absence of membranes and dextran sulfate (DEX. SULF.) as for Fig. 1A. Transcripts were pulsed for 60 min with the buffer adjusted to 80 mM KCl–7.5 mM $MgCl_2$. Vesicular membranes were not present in the reactions in lanes 1 and 2 but were added to the reactions in lanes 3 and 4. Dextran sulfate (20 μg/ml) was added, at the time indicated in Fig. 1A, to the reactions in lanes 2 and 4. When nuclei were formed, transcription was observed in the presence of dextran sulfate (lane 4). (B) Nuclear structure and nuclear import were essentially unaffected by the presence of dextran sulfate (DEX). The DNA was stained with Hoechst H33258, and nuclear import was monitored by the accumulation of a rhodamine-labeled import substrate. Bar, 10 μm.

these reactions, electrophoresed on a denaturing polyacrylamide gel, and analyzed for the presence of specific transcripts by autoradiography. To simultaneously monitor nuclear assembly, the reactions were also examined by fluorescence microscopy for the presence of nuclear membranes encircling the DNA and for import of a fluorescently labeled nuclear import substrate. Since the ability to accumulate this test import substrate is a definitive indication that functional nuclei have formed, it is used here as the most reliable positive criterion for nuclear assembly.

Synthetic nuclei reconstitute most readily around long linear double-stranded DNA templates (26a, 28). In many previous experiments analyzing transport or nuclear assembly, bacteriophage λ DNA was used as the template to maximize nuclear assembly (12, 33, 62, 64, 66–68). In this study, λ templates were sometimes used, but to rapidly obtain long linear DNA from a range of constructs, plasmid DNA containing each gene of interest was ligated into long multimers before use as a template source (see Materials and Methods). When constructs containing the yeast $tRNA_3^{Leu}$ gene in bacteriophage λ DNA or in ligated pUC-based plasmid multimers were compared, the two sources of DNA were found to generate in vitro nuclei which were indistinguishable in terms of nuclear size and the efficiency of nuclear assembly (compare plasmid DNA in Fig. 2B and λ DNA in Fig. 4B). When transcription was first examined, however, we found that the presence of membranes and glycogen caused the tRNA genes in both the λ and ligated plasmid templates to be transcribed poorly compared with their transcription in cytosol alone (data not shown). The concentrations of KCl and $MgCl_2$ were each titrated for optimum pol III transcription. These optimal amounts were greater than what is usually included in a nuclear assembly reaction and were found to be slightly inhibitory to the process of nuclear formation. However, there did not appear to be any deleterious effects of supplementing with these salts after nuclear assembly had taken place. Spermidine, which improved transcription and did not inhibit assembly, was also included. With these adjustments, transcription was found to occur efficiently under conditions of nuclear assembly (Fig. 1B, lane 3). When a DNA template which lacked a pol III gene was used in these reactions, no transcription product was detected after a comparable exposure time (data not shown).

Although the DNA templates encoding tRNA appeared to be efficiently incorporated into nuclei by visual inspection with fluorescence microscopy, we did not know the source of the transcripts produced. Nuclear assembly might repress transcription. Alternatively, templates that had escaped nuclear assembly, although low in number, might be more accessible to the transcriptional machinery and thus be the true source of the transcripts that we observed. Determining this important fact, i.e., whether the origin of the transcripts was nuclear or nonnuclear, was for many years resistant to experimental analysis. A search for nuclear transcripts by using in situ autoradiography proved to be unsuccessful, possibly because of the low sensitivity of this technique (31a). We next tried to digest any DNA not incorporated into nuclei and analyze whether transcripts were generated from the remaining nucleus-enclosed DNA. Unfortunately, when DNase immobilized on microbeads was added to an assembly reaction, the soluble actin present in the extract was found to inhibit the DNase (31a, 52). When micrococcal nuclease-beads were added instead, the assembly of DNA into chromatin and higher-order structures was found to block the action of the nuclease even when no nuclear structure was assembled (31a). Trying a different approach, we searched for a transcriptional inhibitor of RNA pol III which would be unable to enter nuclear pores and which might therefore inhibit transcription from free chromatin, but not that from DNA templates which had been assembled into nuclei.

Entry into nuclei is tightly regulated by nuclear pores such that a nuclear localization signal must be present to direct the entry of large macromolecules (>20 to 40 kDa) through the pores (27, 29, 49, 73). Along with this active transport mechanism, the passive diffusion of smaller molecules into nuclei also occurs. We tested the possibility that the known transcriptional inhibitor, heparin (44, 55), would enter nuclei and serve as a control with access to both the cytosolic and intranuclear RNA pol III machinery. The size of the polyanion heparin (3,000 to 7,000 Da) indicated that it might indeed diffuse into the nucleus. One caveat, however, was that there could be strong ionic interactions of heparin with proteins in the extract (transcription factors being a subset of these) which would

make the effective size of heparin quite large, preventing it from diffusing into the nucleus. When heparin was added to a reaction lacking the vesicular membrane fraction, and therefore lacking nuclei, we found that transcription was fully inhibited (Fig. 1B, lane 2). However, when heparin was added to a reaction in which nuclei had already formed, but before [α-^{32}P]GTP labeling, transcription occurred (Fig. 1B, lane 4). The protection from heparin conferred by the conditions of nuclear formation was specific, since the transcriptional inhibitor α-amanitin, a cyclic octapeptide small enough to freely diffuse into nuclei, prevented transcription even under the conditions of nuclear assembly (Fig. 1B, lane 5). The results were suggestive that heparin is, in fact, excluded from nuclei. Alternatively, heparin might gain access to the nuclei but, because of interactions with the high local concentration of nuclear proteins, be less effective at disrupting transcription under these conditions.

The data presented above indicate that nuclear assembly protects the template from inhibition by heparin. To avoid the question of heparin localization, we tested a higher-molecular-weight polysaccharide, dextran sulfate (M_r, ~500,000), which has chemical properties similar to those of heparin but is clearly too large to enter the nucleus. Dextran sulfate, like heparin, has been used previously to disrupt DNA-protein interactions (3), although its effect on eukaryotic transcription itself had not been tested. When dextran sulfate was added to a transcription reaction containing no nuclei, we found that, like heparin, it effectively inhibited pol III transcription (Fig. 2A, lane 2). In contrast, when dextran sulfate was added to a transcription reaction where the templates had been assembled into nuclei, no inhibition of transcription was observed (Fig. 2A, lane 4). Importantly, neither nuclear structure nor nuclear import was affected significantly by this concentration of dextran sulfate (20 μg/ml), as determined by fluorescence microscopy and transport assays (Fig. 2B).

To determine whether the results obtained with the yeast $tRNA_3^{Leu}$ gene applied more broadly to class III genes, we examined whether a *X. laevis* 5S gene could be transcribed in nuclei assembled in vitro. Since TFIIIA, a factor required for 5S transcription, is often present in limiting concentrations in the egg extract (89), the 5S template (ligated pXls) was routinely preincubated with TFIIIA obtained from an oocyte extract in which this factor is abundant. The 5S DNA was then incubated with glycogen and cytosol for 1 h and finally divided into reaction mixtures to which either the vesicular membrane fraction or buffer was added. After nuclear assembly, reaction mixtures were supplemented with buffer or dextran sulfate and transcription was radiolabeled. The 5S genes were found to be transcribed either in the reaction containing cytosol and buffer (Fig. 3A, lane 1) or under conditions in which the 5S templates were visibly assembled into nuclei (Fig. 3A, lane 3). In the absence of membranes, 5S transcription was inhibited by added dextran sulfate (Fig. 3A, lane 2). However, as was the case with nuclei assembled by using the tRNA template, nuclear assembly around the 5S template provided complete protection from dextran sulfate inhibition (Fig. 3A, lane 4). Thus, different class III genes have the ability to be transcribed under the conditions of nuclear assembly, and additionally, transcription is protected from dextran sulfate when synthetic nuclei are formed.

While the use of templates cloned into plasmid or λ DNA offers the advantage of producing nuclei containing a single type of gene as the sole nuclear transcription template, the use of sperm chromatin as a template for nuclear assembly would be useful for certain transcriptional studies in which analysis in a genomic context is desired. When sperm chromatin is incubated with *Xenopus* egg extract without the addition of any exogenous transcription factors, the primary transcript detected is the SatI transcript, encoded by repetitive satellite DNA (87, 88). As shown in Fig. 3, we found that when sperm chromatin was added to cytosol alone, SatI transcription was inhibited by dextran sulfate (Fig. 3B, lane 2). However, if the sperm chromatin was assembled into nuclei, the transcription of SatI was largely resistant to dextran sulfate inhibition (Fig. 3B, lane 4). This finding eliminated the possibility that something specific to synthetic nuclei formed from cloned DNA templates confers resistance to dextran sulfate and also extended our results to another class III gene.

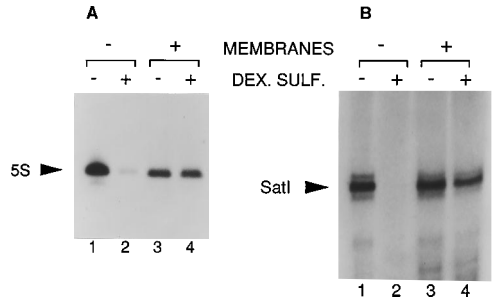

FIG. 3. Transcription of both 5S and genomic SatI genes is protected from dextran sulfate when the templates are assembled into nuclei. (A) Ligated plasmid DNA (pXls) was used in an experiment similar to that of Fig. 1A except that a preincubation step in which TFIIIA was prebound to the DNA to optimize transcription was included. The buffer composition was adjusted to 80 mM KCl–7.5 mM $MgCl_2$–5 μM zinc acetate for the 30-min transcription pulse. Transcription of *Xenopus* somatic 5S was examined as in Fig. 1A in the presence (lanes 3 and 4) and absence (lanes 1 and 2) of membranes. Dextran sulfate (DEX. SULF.) was present in the reactions shown in lanes 2 and 4. The conditions of nuclear formation prevented transcriptional inhibition by dextran sulfate (lane 4). (B) Sperm chromatin was formed into nuclei in the presence of the cytosolic and membrane fractions of an egg extract (lanes 3 and 4) or left as free chromatin by incubation in cytosol alone (lanes 1 and 2). SatI RNA was the primary transcript detected (lanes 1, 3, and 4) (87, 88). When dextran sulfate was added (20 μg/ml) to reactions before transcripts were labeled (lanes 2 and 4), SatI transcription was observed only in reactions in which nuclear formation had occurred (lane 4).

Fusion of membrane vesicles into nuclear envelopes is required to protect RNA pol III transcription from dextran sulfate inhibition. Although the conditions leading to nuclear formation clearly protect transcription from dextran sulfate inhibition, the formal possibility remained that the observed transcription originated from unassembled templates and the membranes were protective for a reason other than nuclear formation (see Discussion). We therefore tested conditions in which all of the components for nuclear formation were present but the process of nuclear envelope assembly was blocked. In the first of such tests, the membrane fraction was treated prior to use with NEM, a sulfhydryl alkylating agent. This procedure has been shown to result in nuclear membrane vesicles which are no longer competent for fusion, although the vesicles can still bind to chromatin (69, 83). When the membrane fraction was treated with NEM, followed by the addition of DTT to inactivate any remaining NEM, and then used in an assembly reaction, nuclei were not formed, as measured by DNA staining and nuclear import (Fig. 4B, NEM panels). Use of the membrane dye DHCC confirmed that although membrane vesicles could bind to chromatin, the fusion required for nuclear formation did not occur in NEM-treated samples (data not shown). Significantly, when dextran sulfate was added to a nuclear assembly reaction containing NEM-treated membranes, tRNA transcription was inhibited (Fig. 4A, lane 6). When, as a control, an aliquot of membranes was treated in

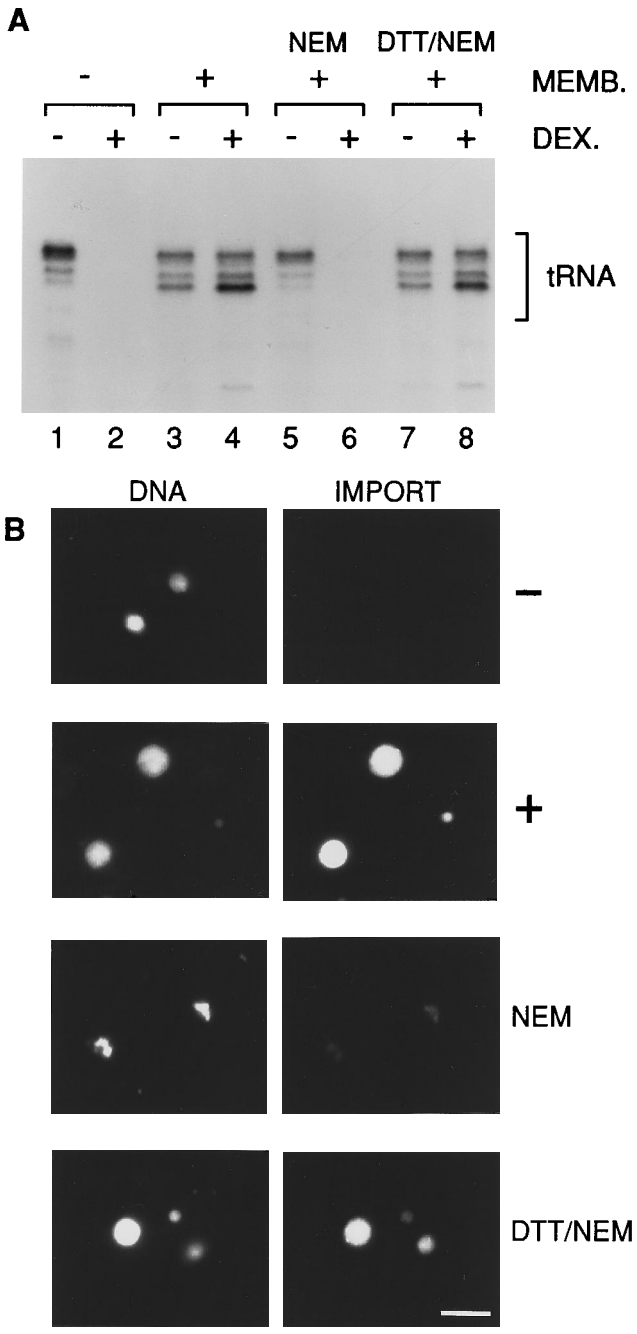

FIG. 4. Membranes must be able to fuse in order to confer protection from dextran sulfate. (A) An aliquot of the vesicular membrane (MEMB.) fraction was incubated for 4 min at room temperature with 3 mM NEM and then incubated with 6 mM DTT for 4 min. Control membranes were treated in parallel with the same chemicals but in the reverse order. Nuclei were assembled from λ DNA, and transcripts were labeled as described for Fig. 1A, with no membranes included (lanes 1 and 2), untreated membranes (lanes 3 and 4), NEM-treated membranes (lanes 5 and 6), or control DTT-NEM-treated membranes (lanes 7 and 8). Dextran sulfate (DEX.; 20 μg/ml) was added as indicated. When the membrane fraction was pretreated with NEM to inactivate the capacity for fusion, the membranes no longer protected transcription from inhibition by dextran sulfate (lane 6). (B) Nuclei formed when untreated (+) or control DTT-NEM-treated membranes were used. No nuclei formed in the absence of membrane (−) or when membranes had been pretreated with NEM. Accumulation of import substrate is shown here to illustrate assembled and functionally competent nuclei. Bar, 10 μm.

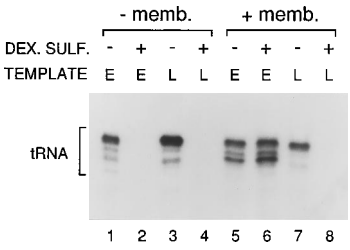

FIG. 5. The order of addition of nuclear components affects resistance to dextran sulfate. The order of addition of the different components needed to form nuclei was altered such that although the reaction mixtures were identical in composition, nuclei were formed only in certain samples. In this experiment, the conditions of nuclear formation were as described for Fig. 1A, with the modifications noted below, and transcripts were labeled for 30 min. In the absence of membranes (memb.), no nuclei formed whether DNA (ligated pPC) was added at an early (E) time point, as was the usual case depicted in Fig. 1A (lanes 1, 2, 5, and 6), or at a later (L) time point just following addition of the transcriptional inhibitor (lanes 3, 4, 7, and 8). When membranes were present and DNA was added early (lanes 5 and 6), nuclei were able to form. In contrast, when DNA was added late to a reaction containing the membrane fraction (lanes 7 and 8), no nuclei were able to form. Dextran sulfate (DEX. SULF.; 20 μg/ml) was added to the reactions indicated, and transcription was found to be resistant to inhibition only in the sample in which where nuclei formed (lane 6).

parallel with the same reagents but in the reverse order so that the membranes remained competent for fusion (Fig. 4B, DTT/NEM panels), nuclear envelope formation occurred and protected against dextran sulfate inhibition (Fig. 4A, lane 8). Inhibition by dextran sulfate was also observed when membrane fusion was blocked by the inclusion of GTPγS in the nuclear assembly reaction (data not shown), further demonstrating that the RNA polymerase active in the reactions is protected from transcriptional inhibition as a result of assembly of a nuclear envelope.

In a second approach to the question of whether membrane fusion and nuclear formation are critical to protection from dextran sulfate inhibition, the order of addition of the nuclear assembly components was altered in such a way that all components were present but nuclei no longer formed. In this experiment, DNA was added either at the beginning of the incubation as was usual or at the time of dextran sulfate addition. In the latter case, no nuclei formed and transcription was sensitive to dextran sulfate inhibition (Fig. 5, lane 8). Thus, even when membranes are present, if their assembly into a nuclear envelope is prevented, transcription is inhibited by dextran sulfate.

Templates must be incorporated into synthetic nuclei to be protected from transcriptional inhibition. As a final test with dextran sulfate, a mixing experiment was performed. An assembly reaction mixture containing a template that had been assembled into nuclei was mixed with a second template that had not (Fig. 6A). Specifically, nuclei were assembled by using the human $tRNA_i^{Met}$ gene template, pΔ3 (2). This template lacks the 3′ termination site of the tRNA gene and thus gives rise to multiple large transcripts which terminate at cryptic stop sites in the pBR322 sequence (2). A parallel reaction with the yeast tRNA template, pPC, was carried out in the absence of membranes. After nuclear assembly had occurred in the pΔ3 reaction with membranes, an aliquot of pΔ3 incorporated into nuclei was combined with an aliquot of pPC incubated without membranes. Once the templates were mixed, transcripts were labeled with [α-^{32}P]GTP in the presence and absence of dextran sulfate. The opposite mixing reaction, in which pPC was assembled into nuclei and pΔ3 was present as free chromatin, was also performed. Consistent with the previous results, when a template was nuclear, transcription was resistant to dextran

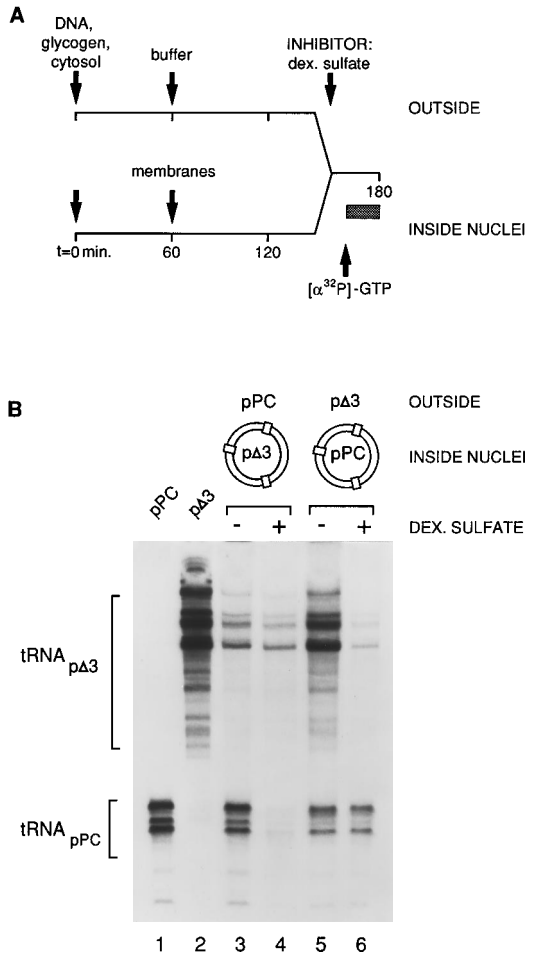

FIG. 6. In mixing experiments, the transcription template must be incorporated into nuclei in order to be resistant to dextran sulfate inhibition. (A) Experimental design of mixing experiments. A ligated plasmid DNA template encoding a transcript of one length was assembled into nuclei essentially as outlined for Fig. 1A. A second ligated plasmid template was incubated identically except that membranes were omitted. Immediately before dextran (dex.) sulfate and radiolabel addition, the two reaction mixtures were mixed such that nuclear and nonnuclear templates (encoding transcripts of different lengths) were present simultaneously during the dextran sulfate inhibition test. The buffer conditions for the transcription pulse were 80 mM KCl and 7.5 mM $MgCl_2$. (B) The transcription products obtained from individual templates transcribed in cytosol alone are shown in lanes 1 and 2 (pPC, lane 1; pΔ3, encoding human $tRNA_i^{Met}$ with a 3′ deletion which results in longer run-on transcripts, lane 2). In lanes 3 and 4, a reaction mixture containing pΔ3 nuclei was mixed with a reaction mixture containing pPC that had been incubated without membranes present. In lanes 5 and 6, a reaction mixture containing pPC nuclei was mixed with a reaction mixture containing free pΔ3. Dextran sulfate (lanes 4 and 6; 20 μg/ml) or buffer (lanes 3 and 5) was added to these mixtures, and transcription was then measured by pulsing with [α-^{32}P]GTP for 30 min. Transcripts encoded by nuclear templates were resistant to dextran sulfate ($tRNA_{p\Delta3}$, lane 4; $tRNA_{pPC}$, lane 6). In the case of pΔ3, transcription levels were lower when nuclear assembly took place. However, we have not observed a consistent depression of transcription upon nuclear formation (see Fig. 1 to 5).

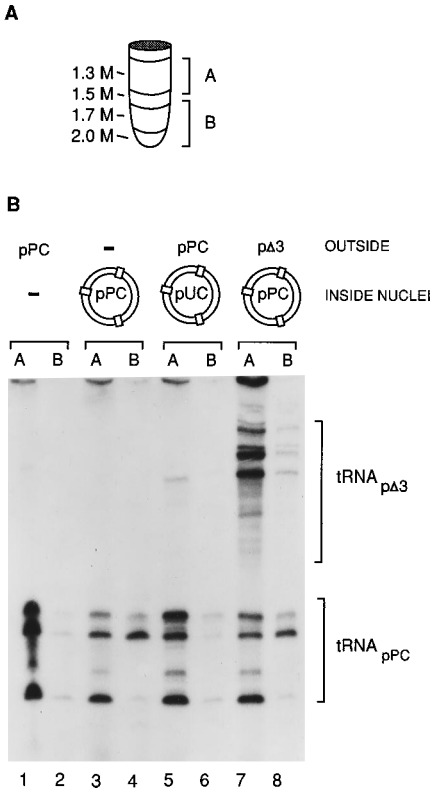

FIG. 7. Transcripts generated from templates incorporated into nuclei can be coisolated with the nuclei. (A) Plasmid templates were incubated under various conditions and then diluted to 1.3 M sucrose and layered onto a gradient of the type illustrated. Following a centrifugation step during which many of the nuclei sediment to the 2.0 M sucrose pad, two fractions (A and B) were collected as indicated. (B) Four reactions were subjected to density centrifugation analysis. The locations of tRNA transcripts were then analyzed by gel electrophoresis. The reactions were set up (see Materials and Methods) such that immediately prior to labeling they contained pPC chromatin (lanes 1 and 2), pPC nuclei (lanes 3 and 4), pPC chromatin and pUC nuclei (lanes 5 and 6), and pΔ3 chromatin and pPC nuclei (lanes 7 and 8). The free glycogen, membrane, and cytosol components remain in fraction A, causing some nonspecific retention of radiolabel in the wells of the A lanes. When pPC was present in nuclei, a substantial proportion of the corresponding transcripts ($tRNA_{pPC}$) sedimented in the lower fraction (B) of the gradient (lanes 4 and 8). The majority of transcripts from the free chromatin templates were absent from the lower fraction (B) ($tRNA_{pPC}$ in lanes 2 and 6; $tRNA_{p\Delta3}$ in lane 8).

sulfate inhibition (Fig. 6B, lanes 3 and 4, upper bands; lanes 5 and 6, lower bands). When the template was not incorporated into nuclei, transcription was inhibited (Fig. 6B, lanes 3 and 4, lower bands; lanes 5 and 6, upper bands). When a different pair of plasmids encoding the *Xenopus* 5S RNA (pXls) and the full-length human $tRNA_i^{Met}$ were assayed, a similar pattern of inhibition was seen (data not shown). These experiments demonstrate that not only must nuclei form to protect transcription from dextran sulfate, but the template in question must be the one incorporated into nuclei for protection to be observed. Of note, transcripts generated with the pΔ3 tRNA construct also indicate that the correct termination sequences are used by pol III within in vitro-assembled nuclei, since deletion of these sequences resulted in run-on transcription products as is the case when this template is free in a transcription extract or in vivo (reference 2 and Fig. 6).

Transcripts can be coisolated with synthetic nuclei. The results described above would predict that early in their biogenesis, transcripts are physically associated with the synthetic nuclei. To test this prediction, nuclei were assembled from ligated pPC plasmid DNA and tRNA transcripts were pulse-labeled with [α-^{32}P]GTP for 30 min. The reaction mix was then diluted with high-sucrose buffer to a final concentration of 1.3 M sucrose and layered onto a step gradient, containing layers of 1.5 and 1.7 M sucrose, and a cushion of 2 M sucrose (Fig. 7A). The densities of the layers were chosen such that the reconstituted nuclei, which are relatively dense, would centrifuge out of the 1.3 M layer, through the other layers, and onto the 2 M cushion. Components of the cytosol and the unincor-

porated membrane vesicles would remain in the 1.3 M layer, while DNA packaged into higher-order chromatin structures would be expected to spin onto the 2 M layer (33). After a short centrifugation, the sucrose gradient was collected in two fractions. The upper fraction (A) included the original sample volume of 1.3 M sucrose plus part of the divider layer of 1.5 M. The lower fraction (B) consisted of the remainder of this divider layer, the 1.7 M layer, and the 2 M cushion. We found that if a pPC tRNA template was not assembled into nuclei, i.e., in reactions which contained no membranes during the incubation, the newly made transcripts remained in fraction A at the top of the gradient (Fig. 7B; compare lanes 1 and 2). In contrast, when the pPC template was assembled into nuclei, a significant proportion of transcripts pelleted in fraction B (Fig. 7B, lane 4). In a control experiment, if pPC template DNA was not assembled into nuclei but instead was mixed into a reaction mixture containing nuclei assembled from carrier plasmid DNA (pUC) before the transcription pulse, the newly made pPC tRNA remained at the top of the gradient (Fig. 7B, lane 5), indicating that the simple presence of nuclei is not sufficient to cause pelleting of transcripts derived from nonnuclear templates. This latter conclusion was also clear from mixing experiments in which pPC templates were formed into nuclei and then mixed with free pΔ3 template DNA before labeling and fractionation on the gradient. In this case, only pPC nucleus-derived tRNA transcripts pelleted into fraction B, while nonnuclear pΔ3-derived transcripts remained in fraction A. These results confirm that synthetic nuclear templates are directing transcription and demonstrate that transcripts can be coisolated with nuclei.

DISCUSSION

RNA pol III transcription takes place in synthetic nuclei. In this study, we have determined that RNA pol III transcription takes place in nuclei assembled in vitro. To examine this question, we first established conditions that were compatible with both nuclear assembly and pol III transcription. Previously, the cytosolic and membrane components of egg extracts were found to be insufficient for effective formation of nuclei from λ DNA (33). The cytosol and membrane fractions obtained by high-speed centrifugation are depleted of a component needed for the efficient formation of higher-order chromatin intermediates. The gelatinous pellet formed during this separation step was found to restore the capability to form nuclei, and in turn, glycogen was found to be the critical component of this fraction (33). The protocol for efficient formation of synthetic nuclei therefore now includes a step in which glycogen, DNA, and high-speed cytosolic supernatant are incubated together prior to the addition of membrane. While this step is necessary for efficient nuclear formation, we initially found that pol III transcription, which is normally robust in *Xenopus* egg cytosol alone, was relatively inhibited by glycogen and, to various degrees, by the membrane fraction. However, we found by experimentation that when spermidine was included in the reaction mixture and when buffer conditions were adjusted to levels optimized for transcription rather than nuclear assembly, efficient pol III transcription was consistently observed under conditions of nuclear assembly. Using this modified protocol, we investigated whether the nuclei were capable of transcription.

To determine whether the transcripts observed originated from newly assembled nuclei or alternatively from templates that had escaped assembly, we searched for an inhibitor of transcription that would fail to gain access to nuclei and thus could be used to distinguish between nuclear and nonnuclear transcription. We found that both heparin and dextran sulfate appear to possess this characteristic. Each strongly inhibits transcription only when templates are nonnuclear. In contrast, α-amanitin can freely diffuse into nuclei and is inhibitory regardless of the presence of nuclei. The effect of dextran sulfate inhibition was not specific to a particular transcript, as the same results were obtained with a number of different RNA pol III-transcribed genes when incorporated into synthetic nuclei. In addition, using sperm chromatin nuclei in which SatI RNA is the primary transcript, we found the same pattern of dextran sensitivity. The latter result demonstrated that protection from dextran sulfate is not due to something specific to nuclei formed from naked DNA, such as the inclusion of glycogen or spermidine, but rather results from nuclear assembly of a template.

We hypothesized that nuclear formation protects transcription from inhibition by the creation of a physical barrier, the nuclear membrane, which prevents certain inhibitors from interaction with the transcription machinery. We assessed the requirements for protection against dextran sulfate inhibition by treating membranes with NEM in order to block their ability to fuse into a nuclear envelope. When membrane fusion was blocked, dextran sulfate inhibited transcription; DTT-NEM-treated control membranes, which did not get alkylated and were thus able to fuse, still conferred protection. We then monitored reactions in which the template and all of the components necessary for nuclear assembly were present but, because of the order of addition, nuclei were not formed. Again in this instance, transcription was inhibited by dextran sulfate. These results indicate that nuclear envelope formation prevents access of dextran sulfate to the proteins active in transcribing pol III genes. As a final test of this conclusion, we mixed reactions mixtures in which one template had been assembled into nuclei and one template had not and then monitored transcription in the presence and absence of dextran sulfate. Transcription directed by the template which had been formed into nuclei was clearly resistant to dextran sulfate, whereas transcription from the template not assembled into nuclei was sensitive to dextran sulfate. This was true of four different templates, each tested inside and outside of nuclei. In sum, this set of experiments confirmed our hypothesis that nuclear formation protects transcription from dextran sulfate and, in turn, confirmed the conclusion that transcription takes place in synthetic nuclei. Of note, since transcription in cytosol alone is well established to produce accurate pol III transcription products (reference 5 and references therein), the fact that major transcription products of the same size were observed in the absence or presence of nuclei with each template tested indicates that pol III transcription in synthetic nuclei is accurately initiated and terminated.

As a final independent assessment of nuclear transcription, we tested whether newly made transcripts are physically associated with nuclei. Since mature transcripts may be immediately exported following transcription and processing, we wished to use transcripts which were not efficiently processed in this system and thus were most likely to remain in association with nuclei. Since the kinetics of intron removal from yeast $tRNA_3^{Leu}$ appears relatively slow in the nuclear transcription reactions, we used this template to determine whether unprocessed transcripts could be coisolated with nuclei in a sucrose step gradient. Such an association was found to be the case. Mixing experiments with nuclear and nonnuclear templates demonstrated that transcripts were coisolated with nuclei only when the corresponding template was nuclear, ruling out the possibilities of nonspecific association of RNA with nuclei or fortuitous colocalization of RNA and nuclei in the gradient. Not all of the potentially nuclear transcripts were found, how-

ever, in the dense sucrose fraction predicted to contain nuclei (fraction B). There are two main possibilities for why recovery in fraction B was not 100%. First, some of the transcripts may exit the nuclei as a result of active export or, alternatively, as a result of possible leakage during processing of the sample. Second, our centrifugation protocol may not quantitatively pellet all of the nuclei, since the synthetic nuclei are often heterogeneous in size and DNA content. We believe that some or all of these possibilities are occurring in our assays. From Fig. 7, lane 3, it can be seen that the faster-migrating mature-length tRNA transcripts (lowest band) are much more highly enriched in the nonnuclear fraction than are unprocessed forms of the tRNA, consistent with potential export of what is likely to be fully processed tRNA. In addition, from experiments in which replicated DNA was radiolabeled and the nuclei were subjected to the same fractionation procedure, we found that a portion of replicated DNA is found in fraction A as well as fraction B (data not shown). Since the replication was dependent on the presence of membranes and thus likely to be occurring in intact nuclei, this finding suggests that not all nuclei pellet into fraction B. Indeed, the proportion of replicated DNA recovered in fraction B (~50%) is similar to the proportion of labeled full-length transcripts recovered in this fraction. While the experiments with dextran sulfate appear to be sufficiently definitive, these results showing coisolation of the transcripts with the nuclei clearly confirm that nuclear templates are being used.

Requirement for a rigorous evaluation of nuclear transcription. Although *Xenopus* egg extracts provide a powerful in vitro approach for studying nuclear function, even slight alterations of the established assembly reaction conditions (whether a change in salt concentration, the addition of a heterologous extract, a decrease in the amount of glycogen, etc.) can severely impair nuclear assembly (33, 33a). Thus, in order to utilize the in vitro nuclear assembly system as an assay for nuclear transcription, it is essential to first establish that nuclear templates are directing transcription. The question is, what provides a rigorous demonstration of this?

Since nuclei will form only if membranes are present, it is tempting to interpret a dependence of active transcription on the presence of membranes as a criterion that nuclear templates are being used. Such an observation is, however, only the first step in establishing that nuclei have formed and are the source of transcriptionally active templates. A serious caveat is that the vesicular membrane fraction could exert an effect on transcription which is unrelated to its ability to form nuclear envelopes. For example, a kinase that regulates the activity of the $p34^{cdc2}$/cyclin B mitotic kinase was recently identified, and this new kinase was found to be present specifically in the membrane fraction of egg extracts (47). This observation highlights the fact that a distinct and different set of enzymatic activities are contributed by the membranes, even in a disassembled state. In addition to a potential effect of such associated proteins, the vesicular membranes are also capable of forming nonnuclear structures that might also affect either transcription initiation or transcript stability. For instance, in nuclear assembly reactions lacking sufficient amounts of glycogen, the DNA is encapsulated in small inert spherical structures rather than being formed into functional nuclei (33). Thus, while a dependence on membranes is suggestive that nuclear formation contributes to transcriptional activation, such a result is open to other interpretations. To distinguish among these possibilities, transcription must be shown to be dependent on not only the components of nuclei but also the completion of nuclear assembly. This can be tested by altering the order of addition of components or by blocking membrane fusion. In either case, transcription can be monitored in a reaction which differentiates between the presence of requisite components for nuclear assembly and the process of nuclear formation itself.

Another way to establish nuclear template usage is to demonstrate that transcription is dependent on a hallmark feature of nuclei. One such feature is the ability to carry out semiconservative DNA replication. At first glance, this seems straightforward since it has been demonstrated that nuclei generated in vitro by using a crude egg extract and naked DNA do indeed replicate their DNA at discrete foci throughout the nucleus in a semiconservative manner (12, 14, 68). However, this property does not exclude the possibility that replication can initiate in a nucleus-independent manner in the absence of membranes, albeit by a different mechanism. This has indeed been seen at times (82a). In past studies concerning replication of DNA in crude egg extracts, much attention was focused on preparing templates that were absolutely free of either RNA fragments that might serve as primers or regions of strand separation that would behave essentially as single-stranded DNA, providing sites for initiation of DNA replication (61). While conditions can be adjusted such that DNA is apparently synthesized solely as a result of semiconservative replication, and hence is dependent on nuclear structures, ideally this should be confirmed to be the case in a study in which DNA replication is used as the proof for nuclear template usage. To provide a definitive demonstration of bona fide semiconservative replication, DNA replicated in the presence of bromo-dUTP could be analyzed by density equilibrium centrifugation (12, 14). Other methodologies, such as blocking replication with the inhibitor aphidicolin or analyzing the extent of the sequences represented in replicated DNA, do not distinguish between replication initiated at gaps in double-stranded DNA versus true semiconservative replication.

Here we have established nuclear template usage by three criteria. First, we have established that defined DNA templates are formed into nuclei under the precise conditions used to measure transcription by assessing nuclear formation with fluorescence microscopy. Second, we have developed an assay for nuclear transcription which relies on a hallmark feature of nuclei: formation of the nuclear envelope, a selective barrier around the DNA. In this assay, RNA synthesis is protected from transcriptional inhibitors that are excluded from nuclei in the presence of nuclear envelope components, but only if these components are assembled into intact nuclear envelopes. Lastly, we have independently demonstrated that transcription occurs in nuclei assembled from a defined template by finding that in vitro-synthesized transcripts coisolate with the corresponding synthetic nuclei.

Transcription in synthetic nuclei: future directions. The observation that pol III transcription occurs in synthetic nuclei provides further insight into the properties of these nuclei. The experimental design which has been utilized integrates the techniques of in vitro transcription with those of in vitro nuclear assembly, making it possible to study aspects of nuclear structure and the specific functions which take place within this environment. By using such an approach, the influence of chromatin formation, higher-order packaging of chromatin, and the assembly of chromatin into subdomains within the nucleus, such as transcriptional or replication domains (13, 34, 36, 37, 39, 40, 53, 60, 81, 85), can now be examined in detail.

Establishing that transcription does take place in synthetic nuclei is the first step in terms of using this system to address these more complex issues. It will now be necessary to determine how much substructural organization is recapitulated in the synthetic nuclei. Several studies have already started to

address this issue. Subnuclear organelles, termed prenucleolar or coiled bodies, have been found by immunofluorescence to be present in synthetic nuclei (10, 11). In addition, early studies of U1 small nuclear ribonucleoprotein localization indicated that this component of the splicing machinery is found within synthetic nuclei (66). Lastly, sites of replication that resemble the endogenous replication foci of somatic nuclei have been identified in nuclei assembled from defined DNA templates (14). The sites of transcription and processing have not yet been analyzed in a similar manner. Such experiments will be important in characterizing the substructural organization of these nuclei and will provide important information as to how closely these in vitro-assembled nuclei resemble their in vivo counterparts.

Transcripts made in synthetic nuclei should in addition prove highly valuable for elucidating the process of RNA export. The components involved in the transport of RNA from its site of synthesis to the nuclear pore and on to the cytoplasm remain largely unknown. To date, progress in this field has been made primarily through the use of oocyte microinjection, fluorescent RNA localization studies, or genetic approaches using yeasts (7, 16, 18–21, 23, 24, 41, 42, 48, 77, 86). However, an in vitro assay for RNA export that is amenable to biochemical dissection has been lacking. The system described here has the potential to be developed into such an assay and would complement the present strategies used to examine this important trafficking pathway.

In summary, in this study various pol III genes were used as templates for transcription. With the exception of TFIIIA, endogenous transcription factors and polymerase were responsible for all of the transcription observed. Pol III transcription offers the advantages of needing no additional extracts to generate transcriptional activity and of synthesizing accurately terminated, directly radiolabeled transcription products. The nucleotide analog bromo-UTP can also be incorporated directly into pol III transcripts (82a), which may prove useful for future applications. Thus, for a range of studies, synthetic nuclei offer the advantages of an in vitro system while retaining many physiological characteristics of normal nuclei. Although no single approach can be used exclusively to dissect complex biological processes, the results described here demonstrate that transcription takes place in synthetic nuclei and suggest that this experimental system may prove powerful for approaching questions concerning the transcription and transport of RNA.

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