Inhibition of RNA polymerase III transcription by a ribosome-associated kinase activity

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ABSTRACT

Ribosomes prepared from somatic tissue of *Xenopus laevis* inhibit transcription by RNA polymerase III. This observation parallels an earlier report that a high speed fraction from activated egg extract, which is enriched in ribosomes, inhibits RNA polymerase III activity and destabilizes putative transcription complexes assembled on oocyte 5S rRNA genes. Transcription of somatic- and oocyte-type 5S rRNA genes and a tRNA gene are all repressed in the present experiments. We find that 5S rRNA genes incubated in S150 extract prepared from immature oocytes exhibit an extensive DNase I protection pattern that is nearly identical to that of the ternary complex of TFIIIA and TFIIIC bound to a somatic 5S rRNA gene. The complexes formed in this extract are stable at concentrations of ribosomes that completely repress transcription, indicating that formation of the TFIII(A+C) complex is not the target of inhibition. Ribosomes taken through a high salt treatment no longer repress transcription of class III genes, establishing that the inhibition is due to an associated factor and not the particle itself. The inhibitory activity released from ribosomes is inactivated by treatment with proteinase K, but not micrococcal nuclease. Preincubation of ribosomes with a general protein kinase inhibitor, 6-dimethylamino-purine, eliminates repression of transcription. Western blot analysis demonstrates that p34[cdc2], which is known to mediate repression of transcription by RNA polymerase III, is present in these preparations of ribosomes and can be released from the particles upon extraction with high salt. These results establish that a kinase activity, possibly p34[cdc2], is the actual agent responsible for the observed inhibition of transcription by ribosomes.

INTRODUCTION

There are two major multigene families that encode 5S rRNA in *Xenopus laevis* (1). The abundant oocyte-type genes are transcribed only during oogenesis and early embryogenesis, while the somatic-type genes are sufficient for ribosome synthesis during the remaining stages of development. Specific developmental repression of the oocyte-type genes is the consequence of a complex interplay between the binding of transcription factors to the internal promoters of these genes and the binding of histone proteins followed by chromatin assembly (2). The stability of these different nucleoprotein complexes ultimately determines the exclusive expression of the somatic-type 5S rRNA genes subsequent to the mid-blastula transition.

The ordered assembly of transcription initiation complexes on the internal promoters of 5S rRNA genes requires initial binding of TFIIIA followed by TFIIIC and, finally, TFIIIB (3). The activity of any of these three factors could potentially contribute to differential expression of the two multigene families. TFIIIA has equal affinity for the promoters of both the oocyte- and somatic-type genes (4). TFIIIC, however, preferentially binds and stabilizes the complex of TFIIIA on somatic 5S rRNA genes (5–7). Thus, limiting amounts of TFIIIA or TFIIIC will contribute to differential expression of the two types of genes. TFIIIB is the target of one or more mitotic kinases that cause a general inhibition of transcription by RNA polymerase III (8,9). Transcription initiation complexes on the oocyte-type 5S rRNA genes appear to be more sensitive to this kinase-mediated repression than complexes formed on the somatic-type genes, suggesting that phosphorylation of TFIIIB may also contribute to the inactivation of the oocyte genes that occurs during embryogenesis (10).

Template exclusion assays have demonstrated the differential stability of transcription complexes on the two types of 5S rRNA genes in activated egg extract (11). While transcription complexes on somatic 5S rRNA genes are resistant to challenge by a second template, complexes on the oocyte genes are measurably less stable. This conclusion is supported by DNase I footprinting experiments that showed selective loss of protection over the promoter region of the oocyte gene upon addition of egg extract. When the activated egg extract was partially fractionated, the inhibitory activity was located in a particulate fraction comprised largely of ribosomes. Purified ribosomes and ribosomal subunits all exhibited the same differential effect on transcription of the two types of 5S rRNA genes (11). The biological significance of this inhibition is not clear. The breakdown of germinal vesicles during completion of meiosis I does represent a time in oocyte maturation when the nuclear and cytoplasmic compartments become mixed, allowing access of cytoplasmic factors to chromatin and nuclear proteins. Indeed, dense clusters of

ribosomes on the basal side of the germinal vesicle invade the nuclear sap during the earliest phases of this process (12).

The experiments reported here were undertaken to delineate the mechanism by which ribosomes preferentially restrict transcription of oocyte 5S rRNA genes. We find that, while the oocyte genes are somewhat more labile, transcription of class III genes is generally inhibited by ribosomes. This effect occurs at concentrations of the particle that do not disrupt the formation or stability of transcription complexes minimally composed of TFIIIA and TFIIIC, suggesting that the target of inhibition is either TFIIIB or RNA polymerase III. Ribosomes taken through a high salt wash no longer inhibit transcription, establishing that this activity is due to an associated factor and not the particle itself. The inhibitor released from ribosomes is sensitive to protease treatment and 6-dimethylaminopurine, implicating a protein kinase activity. Western blot assays demonstrate that p34[cdc2], which has been shown to mediate repression of transcription by RNA polymerase III (9,10), is present in these samples of ribosomes and is, likewise, released from the particles by extraction with high salt.

MATERIALS AND METHODS

Materials

The 7S RNP particle of TFIIIA bound to 5S rRNA was isolated from immature ovaries of *X.laevis* (Nasco, Fort Atkinson, WI) (13). Plasmids containing a single copy of a *X.laevis* oocyte (pXlo316) or somatic (pXP-1) 5S rRNA gene were provided by Dr A.P.Wolffe (National Institutes of Health, Bethesda, MD). The plasmid pXbs115/105 contains the first 115 and last 16 bp of a *Xenopus borealis* somatic 5S rRNA gene joined by a decameric linker (14). This 5S rRNA 'maxigene' produces a 140 nt transcript and was provided by Dr M.T.Andrews (North Carolina State University, Raleigh, NC). A clone of a tRNA[Arg] gene (15) was provided by Dr W.L.Taylor (University of Tennessee, Memphis, TN). Polyclonal antibody to human p34[cdc2] was purchased from Santa Cruz Biotechnology and polyclonal antibody to p70[s6k] was a generous gift from Dr G.Thomas (Friedrich Miescher Institute, Basel, Switzerland).

Extracts and *in vitro* transcription assays

Whole cell (S150) extract was prepared from immature oocytes (primarily stages I–III) according to the method of Glikin *et al.* (16) with the modifications described by Wolffe (5). Transcription reactions contained 20 μl S150 extract and 10 μl T buffer (30 mM Tris–HCl, pH 7.5, 70 mM NH$_4$Cl, 21 mM MgCl$_2$, 1 mM DTT, 30 μM ZnCl$_2$, 10% glycerol) that included 1.5 mM each ATP, CTP and UTP, 75 μM GTP, 5 μCi [α-^{32}P]GTP (ICN) and 30 U RNasin (Promega). The indicated amounts of DNA template and ribosomes were incubated with the extract for 15 min prior to addition of ribonucleoside triphosphates. The mixture was kept at room temperature for 90 min and the reaction stopped by addition of 10 μl proteinase K (1 mg/ml) and 1.5 μl 10% SDS. After 30 min at 37°C, carrier tRNA (5 μg) was added and the sample was sequentially extracted with phenol (twice), phenol/chloroform and chloroform, followed by precipitation with ethanol. The samples were analyzed by electrophoresis on 10% polyacrylamide gels containing 7 M urea followed by autoradiography (5).

Germinal vesicle extract was prepared from stage VI oocytes as described by Birkenmeier (17). Transcription assays (18) were carried out in J buffer (10 mM HEPES, pH 7.4, 70 mM NH$_4$Cl, 7 mM MgCl$_2$, 2.5 mM DTT, 0.1 mM EDTA, 10% glycerol) containing 5–10 μl extract, 200 ng template DNA, 0.5 mM each ATP, CTP and UTP, 0.1 mM GTP and 10 μCi [α-^{32}P]GTP in a final volume of 15–20 μl. After 90 min at room temperature, the reactions were stopped by addition of 85 μl J buffer, 10 μl proteinase K (1 mg/ml) and 1.5 μl 10% SDS. The remaining steps, including analysis by electrophoresis and autoradiography, were identical to those detailed above for assays in S150 extract.

Purification of ribosomes

Ribosomes were purified from *X.laevis* livers according to the method of Martin and Wool (19) and from mature oocytes by the 'low salt' method of Hallberg and Brown (20). A sample of ribosomes was taken through a high salt wash by suspending 80S particles isolated from *X.laevis* liver in TKM buffer (50 mM Tris–HCl, pH 7.8, 25 mM KCl, 5 mM MgCl$_2$, 1 mM β-mercaptoethanol) that had been brought to 850 mM KCl. The sample was layered over a 0.5 M sucrose cushion made in TKM buffer and centrifuged at 4°C in either a Beckman Ty 50.2 rotor at 45 000 r.p.m. for 75 min or a SW60 rotor at 50 000 r.p.m. for 57 min. The upper fraction was drawn off the sucrose cushion and dialyzed overnight against TKM buffer. The sample volume was reduced by packing the dialysis bag in polyethylene glycol (8000) at 4°C. The ribosome pellet was suspended in TKM buffer. The concentrated supernatant fraction and the ribosome fraction were aliquoted into pre-chilled plastic tubes, frozen in a dry ice/ethanol bath and stored at –75°C.

DNase I footprinting

The conditions for DNase I footprinting with purified TFIIIA have been described (21). Footprinting experiments in S150 extract were performed in the same buffer used to measure transcription. Linearized plasmid DNA (200 ng) containing a trace amount of the corresponding end-labeled restriction fragment was incubated with 20 μl extract and the indicated amount of ribosomes for 15 min at room temperature. After addition of ribonucleoside triphosphates, the mixture (final volume of 30 μl) was incubated for an additional 90 min. A 10 μl aliquot was then removed and treated with 2 U DNase I for 1 min. The digestion was stopped by addition of 90 μl DNase stop solution (10 mM Tris–HCl, pH 8.0, 0.3 M NaCl, 5 mM EDTA, 20 μg/ml calf thymus DNA). After addition of 10 μl proteinase K (1 mg/ml) and 1.5 μl 10% SDS the mixture was incubated for 30 min. After sequential extraction with phenol, phenol/chloroform and chloroform, the samples were precipitated with ethanol. The DNA pellet was suspended in loading solution containing formamide, boiled for 4 min and loaded onto 10% sequencing gels (22).

Western blot analysis

Samples of ribosomal proteins (~100 μg) were separated by electrophoresis on 12% polyacrylamide gels containing SDS. Protein was transferred electrophoretically to nitrocellulose membranes at 30 V for 12 h at 4°C (23). After incubation in 100 ml TBST buffer (100 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 1 g non-fat dry milk, the membrane was washed and then incubated in TBST buffer containing the recommended dilution of antibody for 30 min at room temperature. Standard procedures for detection using anti-rabbit IgG–alkaline phosphatase conjugate (Promega) were followed.
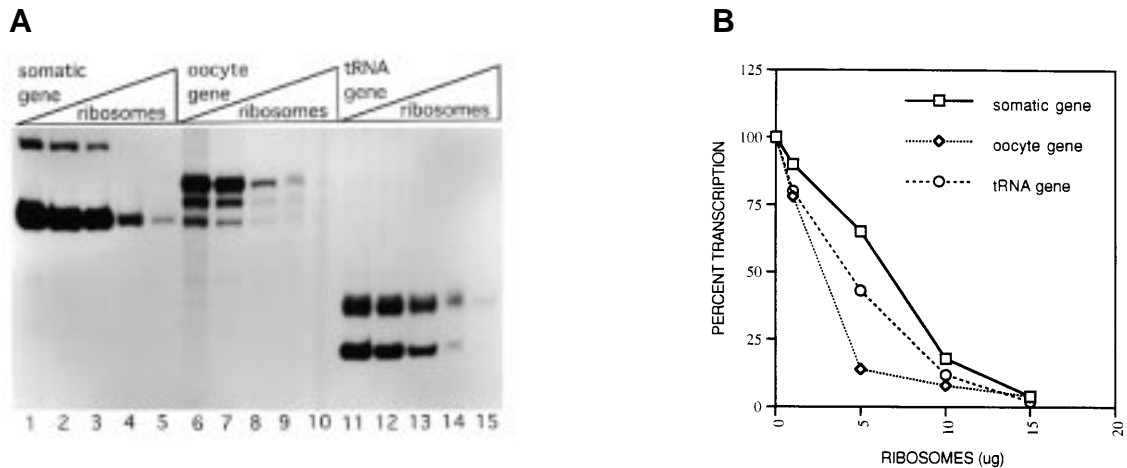
Figure 1. Inhibition of RNA polymerase III transcription by ribosomes. Each reaction (15 µl) contained 8 µl germinal vesicle extract, 200 ng plasmid DNA and the specified amount of purified ribosomes. After incubation for 15 min at room temperature, nucleoside triphosphates, including [α-^{32}P]GTP, were added to initiate transcription reactions which continued for 90 min. (**A**) Samples were analyzed by electrophoresis on 10% polyacrylamide gels containing 7 M urea followed by autoradiography. The amounts of ribosomes per assay are 0 (lanes 1, 6 and 11), 1 (lanes 2, 7 and 12), 5 (lanes 3, 8 and 13), 10 (lanes 4, 9 and 14) and 15 µg (lanes 5, 10 and 15). The oocyte 5S rRNA gene generates three transcripts due to inefficient termination; tRNA^{Arg} yields both a mature transcript and a longer, unprocessed precursor. (**B**) The autoradiograph was scanned with a laser densitometer in order to quantitate the amount of transcription in each assay, which is plotted relative to the amount of added ribosomes.

RESULTS

The addition of activated egg extract to oocyte nuclear extract increases the ratio of transcription of somatic to oocyte 5S rRNA genes (S:O) ~50-fold due to selective inactivation of the oocyte genes (11). Although the egg extract causes a general repression of transcription, template exclusion assays indicate that transcription complexes on the oocyte 5S rRNA genes, but not the somatic-type genes, become selectively destabilized. This differential effect of the extract on the stability of transcription complexes could then account for the increased S:O transcription ratio. When the activated egg extract was fractionated by centrifugation, the resulting particulate fraction contained the activity that represses transcription of the oocyte genes. This high speed pellet is comprised chiefly of ribosomes and glycogen. Subsequent experiments demonstrated that purified ribosomes or the individual small and large ribosomal subunits can inhibit transcription (11). These results potentially have a bearing on the general inhibition of transcription that occurs during germinal vesicle breakdown at the completion of meiosis I and to the low levels of expression of oocyte 5S rRNA genes when transcription resumes at the mid-blastula transition of embryogenesis. Therefore, we undertook experiments to characterize the inhibitory effect of ribosomes on transcription of 5S rRNA genes.

General inhibition of RNA polymerase III transcription by somatic ribosomes

Highly purified eukaryotic ribosomes remain contaminated with other attendant proteins (24,25). In order to eliminate the possibility that the reported effect of ribosomes on transcription of the 5S rRNA genes was actually due to a contaminating, egg-specific activity, we prepared and tested ribosomes from somatic tissue. An earlier electrophoretic analysis of ribosomes prepared from *Xenopus* kidney cells and from eggs did not reveal any detectable difference in the protein composition of particles

from the two types of cells (20). The effect of somatic ribosomes on the transcription of class III genes in germinal vesicle extract is shown in Figure 1. The general inhibition of transcription seen here is analogous to that reported earlier for egg extract and oocyte ribosomes (11). The inhibition of transcription of a tRNA gene (Fig. 1, lanes 11–15) indicates that a factor utilized by both 5S rRNA and tRNA genes is the target of this activity. The transcription assays were repeated using S150 whole cell extract prepared from immature (stages I–III) oocytes. The results (not shown) were identical to those seen in Figure 1. In both extracts transcription of all three genes is inhibited by ribosomes, however, the oocyte 5S rRNA genes are slightly more sensitive. In order to control for the changing concentration of protein in these assays, the effect of identical amounts of either bovine serum albumin or single-stranded DNA binding protein was tested. Neither protein inhibited transcription of the three genes in S150 extract (results not shown), indicating that the observed inhibition is a specific property of ribosomes. The preparations of ribosomes used in these experiments had no detectable DNase or RNase activities that could account for the apparent inhibition.

Stability of the TFIII(A+C) complex

The inhibition of transcription seen in Figure 1 begins when the molar ratio of ribosomes to DNA template is ~5 and is complete at a ratio of 35. This suggests that the particle could be exerting its effect by a direct interaction with the gene or the transcription initiation complex. However, transcription assays in which ribosomes were added after incubation of template DNA with extract exhibited the same degree of inhibition as those in which the DNA was incubated with ribosomes prior to addition of extract. This result suggests that ribosomes do not disrupt transcription complexes on the gene. This conclusion was tested directly in the following DNase I footprinting experiment.

S150 extract prepared from immature oocytes transcribes class III genes efficiently due to the high concentration of transcription

factors, moreover, this extract lacks supercoiling and chromatin assembling activities (5). For these reasons, we felt that it might be possible to measure the stability of transcription complexes on the two 5S rRNA genes in DNase I protection assays using this extract. The conditions used for the footprinting experiments were identical to those used to measure transcription. An extensive region of protection is seen on both the somatic (Fig. 2A) and oocyte (Fig. 2B) 5S rRNA genes in this extract and is compared with the footprint of TFIIIA (lanes 7). Protection on both genes extends from nt 15/17 to ~115, whereas the TFIIIA binding site encompasses nt 45–96. Footprinting experiments using whole cell or nuclear extracts prepared from late stage oocytes generally show protection patterns resembling that of TFIIIA alone (18,26–28). The protection seen here in extract from immature oocytes, however, is very similar to that determined for the ternary complex of TFIIIA and TFIIIC bound to an *X.laevis* somatic 5S rRNA gene (29). In the latter case, a pattern of continuous protection extends from roughly nt 15 to the 3′-end of the TFIIIA footprint at position 96. Similarly, the footprint of human TFIIIC on a *Xenopus* 5S rRNA gene begins at nt 25 and extends up to the region protected by TFIIIA (30). The only difference between the footprints in Figure 2 and that reported for the *Xenopus* TFIII(A+C) complex is the additional protection seen at nt 96–115 in the former. Notably, the protection pattern we detect in S150 extract is comparable with the *Saccharomyces cerevisiae* TFIII(A+C) core complex in which the 3′ boundary of the footprint extends out to approximately nt 120 (31).

We cannot determine the precise composition of the complex residing on the 5S rRNA genes from these footprints, specifically, whether TFIIIB is present. Indirect evidence suggests that *Xenopus* TFIIIB binds at a site ~35 bp upstream of the gene (32). However, unlike the case with the yeast factor, DNase I footprinting experiments have failed to yield a protection pattern that can be attributed to vertebrate TFIIIB (32). Nonetheless, by comparison with the results of Sturges *et al.* (29), the complex formed in S150 extract must be minimally comprised of TFIIIA and TFIIIC.

The addition of increasing amounts of ribosomes to concentrations exceeding those needed to inhibit transcription did not alter the footprints on either 5S rRNA gene (Fig. 2). Likewise, preincubation of template DNA with the ribosome fraction prior to mixing with the S150 extract did not interfere with formation of the complexes on either gene nor did it change the appearance of the protection pattern. Since the degree of inhibition by ribosomes is similar for the three genes tested (Fig. 1), the target must be a factor required for transcription of all class III genes and not TFIIIA, which is specific for 5S rRNA genes. The results in Figure 2 support this conclusion by showing that a minimal complex of TFIIIA and TFIIIC is not disrupted by ribosomes or a ribosome-associated activity. The most likely target of inhibition, then, is either RNA polymerase III or TFIIIB, which is the actual activation factor for this polymerase (33).

Unlike the results in Figure 2, footprints representing putative transcription complexes in germinal vesicle extract prepared from mature (stage VI) oocytes are quite similar to that for TFIIIA alone (18,34). Addition of activated egg extract or a ribosome-enriched particulate fraction appeared to eliminate this protection selectively from the oocyte-type genes (11). This presents the possibility that binding of TFIIIA to initiate formation of transcription complexes on these genes was specifically inhibited. However, we have carried out mobility shift assays which
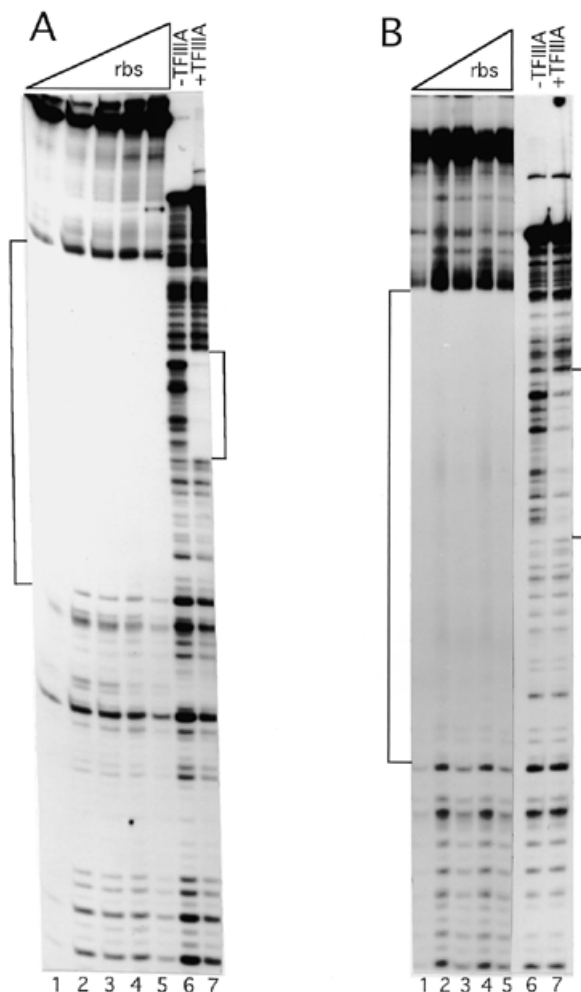

Figure 2. DNase I footprinting assays in oocyte S150 extract. Each reaction (30 µl) contained 20 µl S150 extract prepared from stage I–III oocytes, 200 ng linearized plasmid DNA labeled on the non-coding strand and the specified amount of ribosomes. After incubation for 15 min at room temperature, nucleoside triphosphates were added and the incubation continued for an additional 90 min. Samples were then digested with DNase I for 1 min and prepared for analysis by electrophoresis on sequencing gels. Lanes 1–5 contained 0, 5, 10, 15 and 20 µg ribosomes, respectively. Lanes 6 are digestions of DNA alone and lanes 7 contain 26 nM TFIIIA. (**A**) Somatic 5S rRNA gene and (**B**) oocyte 5S rRNA gene.

demonstrate that TFIIIA–5S rDNA complexes are not disrupted at concentrations of ribosomes exceeding those that inhibit transcription (results not shown). This result is in accord with the observed inhibition of transcription of the tRNA gene (Fig. 1), which does not utilize TFIIIA.

Washed ribosomes do not inhibit RNA polymerase III transcription

Eukaryotic ribosomes contain ~80 proteins; however, the exact number is not certain because the individual proteins cannot be assayed for function and reconstitution of the particle has not been possible (35). Moreover, preparations of ribosomes frequently contain other associated cytoplasmic proteins (24). We analyzed
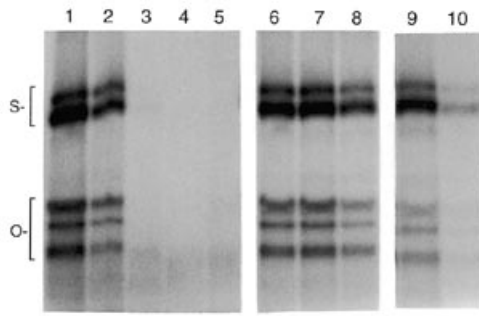
Figure 3. Salt-washed ribosomes do not inhibit transcription by RNA polymerase III. Each transcription assay (20 μl) contained 5 μl germinal vesicle extract, 20 ng plasmid DNA carrying a somatic 5S rRNA maxigene and 80 ng plasmid DNA carrying an oocyte 5S rRNA gene. Lanes 1–5 contain 0, 1, 5, 10 and 25 μg ribosomes, respectively. Lanes 6–8 contain, respectively, 5, 10 and 25 μg ribosomes extracted with TKM buffer containing 850 mM KCl and pelleted by centrifugation through a sucrose cushion. Lanes 9 and 10 contain 5 and 10 μl concentrated supernatant fraction containing material released from the salt-treated ribosomes, respectively. Brackets indicate transcripts from the somatic (S) and oocyte (O) genes.

Figure 4. The ribosome-associated inhibitor is sensitive to treatment with protease. Standard transcription assays contained the following additions: lane 1, J buffer; lane 2, 10 ng proteinase K and 8 mM AEBSF in J buffer; lane 3, 10 ng proteinase K in J buffer; lane 4, 0.1 U MNase, 0.2 mM $CaCl_2$ and 0.8 mM EGTA in J buffer; lane 5, 2.5 μl supernatant; lane 6, 5 μl supernatant; lane 7, 10 μl supernatant; lane 8, 0.1 U MNase, 0.2 mM $CaCl_2$ in J buffer; lane 9, 10 μl supernatant kept for 15 min at 30°C; lane 10, 10 μl supernatant treated with 0.1 U MNase, 0.2 mM $CaCl_2$ in J buffer at 30°C for 15 min followed by addition of 0.8 mM EGTA and further incubation at 4°C for 30 min; lane 11, 10 μl supernatant treated with 10 ng proteinase K at 30°C for 15 min followed by addition of 8 mM AEBSF and further incubation at 4°C for 30 min. Brackets indicate transcripts from the somatic (S) and oocyte (O) genes. The concentration of protein in the supernatant fraction was too low to measure accurately.

the protein composition of our preparation of ribosomes by electrophoresis on SDS–polyacrylamide gels. The electrophoretic pattern of Coomassie stained proteins was similar to the well-characterized profile of rat 80S proteins (19) and did not indicate any appreciable contamination. A sample of these ribosomes was suspended in high salt buffer (850 mM KCl) and then pelleted by ultracentrifugation through a sucrose cushion; this treatment can remove weakly associated proteins without disrupting the integrity of the particle (19,24). We determined that these conditions are not sufficient to dissociate *Xenopus* 80S ribosomes into their constituent subunits. Additionally, the profile of proteins stained by Coomassie blue was not detectably changed by this procedure. These ribosomes were then assayed for their effect on transcription of class III genes. We have added both an oocyte 5S rRNA gene and a somatic 5S rRNA 'maxigene' to these transcription mixtures in order to compare directly the response of the two types of genes under the same assay conditions. Salt-washed ribosomes do not inhibit transcription of either somatic- or oocyte-type 5S rRNA genes (Fig. 3) or a tRNA^Arg gene (results not shown). Whereas inhibition of transcription is observed at <1 pmol untreated ribosomes, no inhibition could be detected in the presence of a 10-fold higher concentration of the same ribosomes taken through the high salt wash. The supernatant fraction, containing material released by the salt extraction, was concentrated ~7-fold and found to inhibit transcription (Fig. 3, lanes 9 and 10). These results indicate that, rather than the particle itself, some activity associated with ribosomes mediates inhibition of transcription by RNA polymerase III. As a consequence of these results, we prepared ribosomes from *Xenopus* oocytes using the criterion of purity (A_{259}/A_{236} = 1.58 ± 0.04) described by Hallberg and Brown (20); this procedure contains several additional steps compared with the one used to isolate ribosomes from liver tissue. The oocyte ribosomes purified by this method did not affect transcription of the 5S rRNA and tRNA genes (results not shown), supporting the notion that the inhibition observed before (11) and in the present study is not due to the particle, but rather to an associated factor.
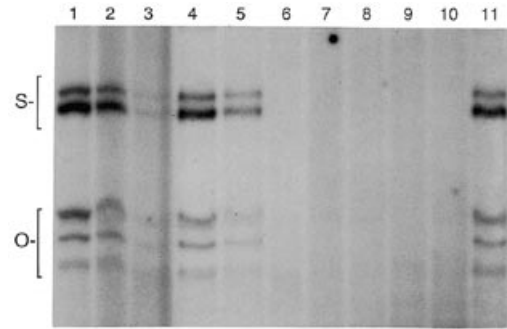
We have determined that transcription of class III genes in germinal vesicle extract is sensitive to the addition of exogenous RNA, therefore, we considered the possibility that the salt extraction removed remnant RNA from the ribosome sample. The nature of the ribosome-associated inhibitor was determined by treating the supernatant from the salt extraction with either micrococcal nuclease (MNase) or proteinase K and then testing these samples in transcription assays (Fig. 4). An aliquot of the supernatant was incubated with 0.1 U MNase for 15 min at 30°C in the presence of 0.2 mM $CaCl_2$. This amount of nuclease was sufficient to eliminate a trace amount of radiolabeled 5S rRNA added to an identical control reaction. The nuclease was inactivated by addition of EGTA (final concentration 0.8 mM) and an additional incubation at 4°C for 30 min. This sample retained its inhibitory activity (Fig. 4, lane 10); a mock control of buffer treated in the same way had no effect on transcription (Fig. 4, lane 4). Another aliquot of the supernatant was incubated with 10 ng proteinase K for 15 min at 30°C. The digestion was stopped by addition of 4-(2-amino-ethyl)benzenesulfonyl fluoride (AEBSF) and an additional incubation at 4°C for 30 min. This treatment abolished the inhibitory activity of the sample (Fig. 4, lane 11), establishing that a protein released from ribosomes is responsible for the repression of transcription.

Treatment of ribosomes with 6-dimethylaminopurine eliminates inhibition of transcription

The p34^cdc2–cyclin B complex (maturation/mitosis promoting factor, MPF) can trigger repression of transcription by RNA polymerase III in interphase egg extract (9). This effect is due to phosphorylation of TFIIIB. Moreover, Leiss *et al*. (36) have shown that a substantial amount of p34^cdc2–cyclin B complex is associated with 80S ribosomes in activated *Xenopus* eggs and can be released from the particle by treatment with high concentrations of salt. Their observation parallels the experiment in Figure 3 showing that
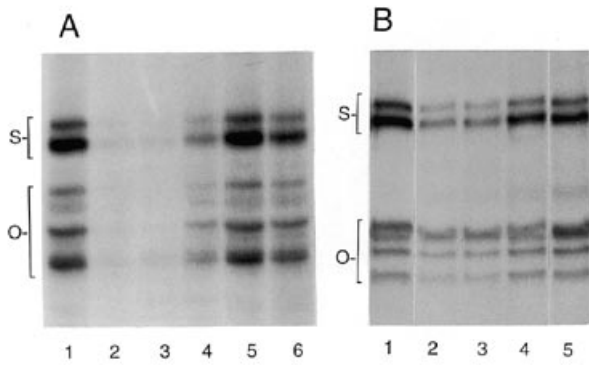
Figure 5. 6-Dimethylaminopurine reverses inhibition of transcription. (**A**) Ribosomes (25 µg) were incubated with the indicated concentration of DMAP for 20 min at room temperature. The ribosome sample was then added to a standard transcription assay programmed with both somatic and oocyte 5S rRNA genes (1:4 molar ratio). Lane 1, no additions; lanes 2–6, ribosomes treated with 0, 1, 2, 5 and 10 µM DMAP, respectively. (**B**) Supernatant material released from salt-extracted ribosomes was incubated with the indicated concentration of DMAP for 20 min at room temperature and then, likewise, added to transcription assays. Lane 1, no additions; lanes 2–5, supernatant fraction treated with 0, 0.5, 1 and 10 µM DMAP, respectively. Brackets indicate transcripts from the somatic (S) and oocyte (O) genes.

Figure 6. Western blot assay for p34[cdc2] in ribosomes. Samples containing 100 µg protein from untreated ribosomes (lane 2) or salt-extracted ribosomes (lane 3) were separated on a SDS–polyacrylamide gel, transferred to a nitrocellulose filter and probed with antibody prepared against p34[cdc2]. Lane 1 contains prestained molecular weight standards of the indicated mass (kDa).

salt-washed ribosomes no longer repress transcription by RNA polymerase III. In order to determine whether a ribosome-associated kinase activity mediates the observed inhibition of transcription, we treated ribosomes with the general kinase inhibitor 6-dimethylami-nopurine (DMAP) and then tested the effect of these ribosomes on transcription of 5S rRNA genes. DMAP is able to fully reverse the inhibitory effect of ribosomes (Fig. 5A). Interestingly, at high concentrations (≥10 µM) DMAP itself begins to inhibit trans-cription, presumably by acting as a competitive inhibitor with respect to ribonucleoside triphosphates. We also tested the effect of DMAP on the material released from ribosomes by salt extraction (Fig. 5B) and found that inhibition of transcription, likewise, was attenuated. These results establish that a kinase activity associated with ribosomes can repress transcription of class III genes.

On the basis of the preceding results, we tested for the presence of p34[cdc2] in our preparations of ribosomes by western blot analysis (Fig. 6). A protein band with a molecular mass of ~34 kDa reacts with the antibody. Two additional bands of 31 and 22 kDa are also present, which we assume represent proteolytic fragments of the kinase. Salt-washed ribosomes, however, contain no polypeptides that react with the p34[cdc2]-specific antibody (lane 3). Release of the kinase by treatment with high salt, then, coincides with removal of the inhibitor from the ribosomes. We also used a western blot assay to test for the presence of p70[s6k] in our preparation of ribosomes. This kinase phosphorylates multiple sites within the C-terminus of ribosomal protein S6 in response to various hormones and growth factors (37,38). Phosphorylation of S6 during oocyte maturation remains essentially constant (39). However, the level of S6 phosphorylation can be increased by injection of MPF into stage IV oocytes or by progesterone-induced maturation of stage VI oocytes (39). No reaction of a polyclonal antibody specific for p70[s6k] could be detected with our preparations of ribosomes (results not shown).

Although the western assay establishes the presence of p34[cdc2] in these preparations of somatic ribosomes, just as this kinase is also found associated with ribosomes in egg extract (36), phosphorylation assays indicate that one or more other kinases are
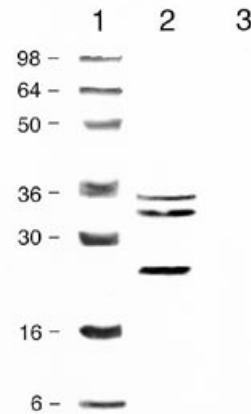
also present. Histone H1 is a diagnostic substrate for p34[cdc2]. When ribosomes were incubated in J buffer with histone H1 and [γ-^{32}P]ATP, only modest amounts of histone H1 phosphorylation were observed, while at least two other proteins in the sample became phosphorylated to a relatively greater degree (results not shown). We repeated this assay using the supernatant fraction from ribosomes extracted with salt and obtained the same result. It is clear that biochemical fractionation will be required to establish whether any other ribosome-associated kinase, in addition to p34[cdc2], can inhibit polymerase III transcription.

DISCUSSION

We have determined that the observed repression of RNA poly-merase III transcription by ribosomes (11) is due to an associated kinase activity and not the particle itself. An immunochemical assay establishes that p34[cdc2] kinase is present in our preparations of ribosomes and is stripped from the particle using the same conditions that release the inhibitory activity. Gottesfeld and co-workers have determined that p34[cdc2]–cyclin B complex mediates the general repression of transcription by RNA polymerase III via phosphory-lation of TFIIIB (8–10,40). It must be noted, however, that p34[cdc2] does not appear to be the sole kinase activity in these preparations of ribosomes, so it remains possible that another kinase contributes to the observed inhibition of transcription.

Earlier DNase I footprinting experiments in oocyte nuclear extract demonstrated that a protection pattern similar to that of TFIIIA, ascribed to a complete transcription complex, was lost from oocyte 5S rRNA genes upon addition of activated egg extract (11). If some component of this extract promotes disruption of transcription complexes from oocyte 5S rRNA genes, it is distinct from the ribosome-associated kinase activity. Our footprinting experiments in S150 extract establish that formation of complexes minimally composed of TFIIIA and TFIIIC is not perturbed at concentrations of ribosomes that completely inhibit transcription. Moreover, mobility shift assays demonstrate that the concentration of ribo-somes needed to interfere with binding of TFIIIA to the 5S genes is >10-fold higher than that needed to repress transcription. These results point to TFIIIB as the target of the ribosome-associated

kinase and are in accord with the repressive effect of p34[cdc2] on the activity of this factor (9). It must be noted that, despite indirect evidence that TFIIIB binds immediately upstream of 5S rRNA genes, it has not been possible to detect the presence of the vertebrate factor in initiation complexes using footprinting assays (32). Moreover, it is not known how phosphorylation inhibits TFIIIB activity, i.e. whether this modification affects its assembly into the initiation complex, its transcriptional activation activity or both.

We detect modest differences in repression of the two types of 5S rRNA genes; the oocyte-type genes are only slightly more sensitive to inhibition at intermediate concentrations of added ribosomes. The addition of p34[cdc2]–cyclin B complex to an interphase egg extract repressed transcription of a somatic 5S rRNA gene ~80%, whereas transcription of both an oocyte 5S rRNA gene and a tRNA gene were reduced >95% (10). If, however, cyclin B1 was added prior to the template DNA, transcription of all class III genes was inhibited equally. Apparently, factors assembled into transcription complexes on the two types of 5S rRNA genes, unlike free factors, are repressed to somewhat different degrees by the mitotic kinase. Wolf *et al.* (10) have speculated that this effect may contribute to the switch in 5S rRNA gene expression that occurs during oocyte maturation. Since the rate of phosphorylation of TFIIIB may be significantly faster than the rate of transcription *in vitro*, the difference observed by us and others (10) between the somatic- and oocyte-type genes may actually be an underestimate compared with processes that occur *in vivo*.

Progesterone-induced meiotic maturation of *Xenopus* oocytes triggers activation of p34[cdc2]–cyclin B and concomitant breakdown of the germinal vesicle. At this time transcription is repressed even though transcription factors are still abundant in the cell (1). The simple accessibility of activated p34[cdc2] kinase to TFIIIB (as well as other targets) could account for the general repression of transcription that occurs at this stage of development (9,10). The question that arises is whether the subcellular localization of p34[cdc2] with ribosomes is germane to this process. Leiss *et al.* (36) found evidence that a factor in the ribosomal fraction of *Xenopus* eggs is required for activation of the kinase activity of the p34[cdc2]–cyclin B complex. Alternatively, or in addition, the association of activated p34[cdc2] with ribosomes may simply facilitate rapid delivery of the kinase to the nucleus. Cytochemical and ultrastructural analyses have shown that breakdown of the germinal vesicle membrane begins on its basal side, where ribosomes are densely clustered in basophilic bodies (12). The nuclear sap is invaded with ribosomes upon initial rupture of the nuclear membrane. Therefore, nuclear transcription factors are immediately exposed to a high concentration of ribosomes and their associated proteins at a time that coincides with repression of transcription. Thus, transcriptional inactivation may not be a passive process resulting from simple mixing of nuclear and cytoplasmic components, but rather may be expedited by the subcellular localization of p34[cdc2]–cyclin B complex with ribosomes.

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