Transcription of Class III Genes: Formation of Preinitiation Complexes

Andrew B. Lassar, Paul L. Martin, Robert G. Roeder

Summary. Class III genes require multiple cellular factors for transcription by RNA polymerase III; these genes form stable transcription complexes, which in the case of *Xenopus* 5*S* genes are correlated with differential expression in vivo. The minimal number and identity of the factors required to form both stable and metastable complexes on three class III genes (encoding, respectively, 5*S* RNA, transfer RNA, and adenovirus VA RNA species) were determined. Stable complex formation requires one common factor, whose recognition site was analyzed, and either no additional factors (the VA gene), a second common factor (the transfer RNA gene), or a third gene-specific factor (the 5*S* gene). The mechanism of stable complex formation and its relevance to transcriptional regulation were examined in light of the various factors and the promoter sequences recognized by these factors.

Our present understanding of the mechanisms and regulation of transcription of eukaryotic genes is limited, but the development of cell-free systems that accurately transcribe exogenous (purified) genes has begun to provide significant information about both the DNA sequences and the cellular (protein) factors involved in these processes. Although these studies [reviewed in (*1*)] have included analysis of genes transcribed by RNA polymerases I (encoding the large ribosomal RNA's), II (encoding messenger RNA's), and III (encoding small structural RNA's), this article is restricted to our analysis of well-characterized class III genes (those transcribed by RNA polymerase III) encoding transfer RNA (tRNA), 5*S* RNA, and adenovirus VA RNA (*2*).

Analysis of promoter sequences by mutagenesis of cloned genes showed that a 34-nucleotide stretch within the 5*S* gene is necessary and sufficient for initiation of transcription (*3, 4*), and that several tRNA genes and the adenovirus VA I gene contain internal promoter elements in addition to upstream modulatory sequences [reviewed in (*5, 6*)]. More detailed studies revealed that the tRNA promoter region contains two noncontiguous stretches of DNA, termed A and B blocks, which are also present in the VA gene promoter (*7, 8*). The 5*S* RNA gene also appears to contain two separable elements, one homologous to the A block of tRNA genes and the other specific to 5*S* genes (*6*). The promoter elements for the *Xenopus* $tRNA_1^{Met}$ and human adenovirus VA I genes (representative of genes containing A and B blocks) and the *Xenopus* 5*S* RNA gene are depicted in Fig. 1.

The factors that are required, along with RNA polymerase III, for transcription of purified class III genes have been analyzed by chromatographic fractionation of crude cellular extracts. Our earlier work (*9–11*) established, for both amphibian and human cells, that two factors (designated IIIB and IIIC) are necessary for transcription of the tRNA and VA RNA genes, whereas 5*S* genes require these same factors plus a third gene-specific factor (IIIA). Despite extensive purification (*12*), it has not yet been possible to distinguish individual IIIB and IIIC factors specific for one subgroup of class III genes. Presumably, one or more of these factors or RNA polymerase III (or a combination) interacts with the conserved sequence blocks in the tRNA and VA RNA gene promoters and with the common element present in the 5*S* gene. In the case of factor IIIA, a single protein of 38,000 daltons has been purified to homogeneity (from *Xenopus* oocytes) and shown to interact, in the absence of other factors, with the internal control region of both amphibian (*10*) and mammalian (*13*) 5*S* RNA genes, thus indicating its key role as an initiation factor. This factor also interacts stoichiometrically with 5*S* RNA in oocytes, apparently to stabilize the stored 5*S* RNA (*14, 15*); this interaction, plus the demonstrated potential of 5*S* RNA to inhibit factor IIIA function in vitro (*15*), suggests that, in the cell, 5*S* gene transcription in the presence of limiting amounts of this factor may be subject to autoregulation, may be restricted to previously activated genes, or both.

Before initiation of transcription, stable complexes form between purified genes and transcription factors in crude extracts, as demonstrated by Bogenhagen *et al.* (*16*) for 5*S* genes. Such complexes persist for many rounds of transcription, even in the presence of other competing templates. Evidence that such complexes exist in vivo was provided by Parker and Roeder (*17*), who showed that chromatin isolated from immature oocytes contains (in a stable association) all the factors necessary to promote accurate transcription of endogenous oocyte-type 5*S* genes by a purified RNA polymerase III. Bogenhagen *et al.* (*16*) subsequently demonstrated that somatic cell chromatin retains, in the presence of exogenous factors and polymerase, the 5*S* gene specificity (repression of oocyte genes and expression of somatic genes) imposed in vivo. The establishment of stable complexes within an otherwise repressive chromatin structure may thus represent a key step in gene activation and may provide a means for the maintenance and propagation of a specific set of activated genes, even in the absence of other factors that may have been essential for their formation (within chromatin).

The formation of stable transcription complexes is a general feature of class III genes, and possibly of class I and class II genes as well (*18, 19*). Because of the general significance of such complexes, for understanding both mechanistic and regulatory aspects of transcription, we examined the requirements for their formation on 5*S* RNA, tRNA, and VA RNA genes in purified DNA templates. By using separated transcription factors from human cells, we determined the minimal number and identity of the components necessary for formation of stable complexes. We also demonstrated less stable interactions as well as a promoter site interaction for a factor required by all class III genes.

The authors are at the Laboratory of Biochemistry and Molecular Biology, The Rockefeller University, New York 10021. Some of the work described in this article was carried out in the Department of Biochemistry, Washington University School of Medicine, St. Louis, Missouri 63110.

Complexes on the 5S RNA Gene

Factors A and C are both necessary and sufficient for a stable complex. The RNA polymerase III factors from human and amphibian (*Xenopus*) cells are chromatographically similar (*9, 11*) and mediate, in homologous and heterologous combinations, accurate transcription of both amphibian and mammalian class III genes (*13*). Given this evolutionary conservation of structural and functional features of these factors, we used the more readily separated human factors to investigate the interactions of these factors with DNA (*20*). Our analysis of DNA–transcription factor complexes is based on the assay described by Bogenhagen *et al.* (*16*). This assay monitors the ability of one gene, when incubated with limiting amounts of an extract, to exclude transcription of a second (functionally equivalent) gene added subsequently (with remaining substrates). This assay is equivalent to the two-step protocol diagramed in Fig. 2, with all factors added in the first step. The preferential transcription of the first gene during the secondary incubation indicates the stable interaction of a limiting component or components in the first incubation.

Our initial experiments demonstrated that prior incubation of a *Xenopus* 5S gene with unfractionated human cell extracts (two-step protocol of Fig. 2) results in the formation of complexes that preclude transcription of a second 5S gene (data not shown), in agreement with the data on oocyte extracts (*16*). That this result can be reproduced in a system reconstituted with separated transcription factors (A, B, C, and RNA polymerase III) is shown in Fig. 3. As indicated, prior incubation of all factors with a 5S maxigene [an insertion mutant that retains a fully functional promoter (*21*)] results in preferential transcription of this gene during subsequent incubation with the wild-type 5S gene (lane 2 in Fig. 3); the presence of both templates during the first incubation results in equivalent levels of transcription from each (lane 1 in Fig. 3). Other control experiments indicate that prior incubation of the factors with pBR322 (containing no 5S sequences) does not exclude transcription of 5S genes added subsequently (lane 2 in Fig. 4, a and b). For convenience we refer to the partially purified fractions as factors; although further purification has failed to reveal additional multiplicity (*12*) this possibility cannot yet be excluded.

To determine which of the individual factors are necessary to form a stable

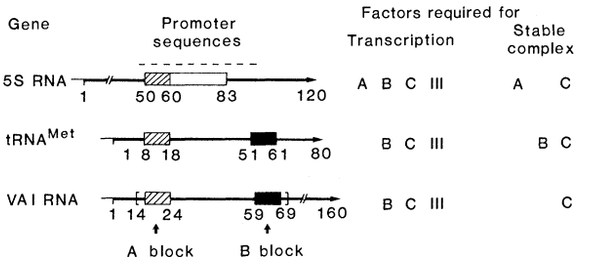

Fig. 1. Summary of transcription factor requirements, promoter regions and homologous sequences in the *Xenopus borealis* somatic 5S gene, the *Xenopus laevis* $tRNA_i^{Met}$ gene and the adenovirus VA I gene. Promoter regions are represented by rectangles, the A sequence block by a striped rectangle and the B sequence block by a black rectangle. Numbers indicate nucleotides downstream from the 5′ end of the mature transcript. The dashed line above the 5S gene indicates the region protected from deoxyribonuclease I digestion by *Xenopus laevis* factor IIIA. Brackets in the VA I gene designate the extent of the promoter as defined by deletion analysis. References to the promoter regions are: 5S (*3, 4, 10*); $tRNA_i^{Met}$ (*34*); VA I (*8, 35*).

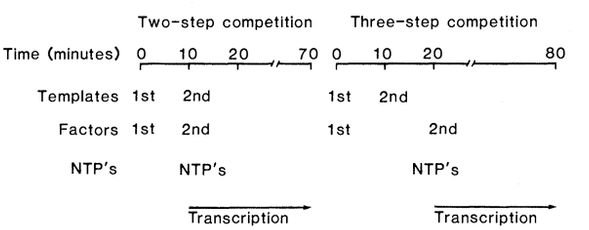

Fig. 2. Diagram of two-step and three-step incubation-competition protocols; *NTP*'s, nucleoside triphosphates.

complex on the *Xenopus* 5S RNA gene, we first incubated subsets of the chromatographically separated factors (legend to Fig. 3) with the 5S maxigene and added the remaining factors and nucleoside triphosphates with the secondary wild-type 5S gene (two-step incubation-competition in Fig. 2; but see below). As shown in Fig. 3, prior incubation of the primary template (maxigene) with individual factors (plus RNA polymerase III) (lanes 3 to 5) or with pairwise combinations A and B (lane 6) or B and C (lane 8) led to equivalent levels of transcription of the primary and secondary genes: this was the same as was observed when both templates were present during the first incubation with all factors (lane 1). However, prior incubation of the primary template with factors A and C, either with (lane 7) or without (lane 9) RNA polymerase III diminished transcription of the secondary gene to the same extent as observed when all three factors were present in the first incubation (lane 2). Thus, factors A and C, but not RNA polymerase III or factor B, are necessary for stable complex formation. To rule out the unlikely possibility that a trace amount of B activity contaminating factors A and C might have participated in stable complex formation, we subjected these factors to further purification (legend to Fig. 3). These preparations, devoid of any detectable factor B activity, are sufficient, when incubated with a 5S gene, to reduce transcription of the secondary gene (lane 11).

The above experiments do not distinguish between the possibility that both factors A and C remain bound to the first template and the possibility that one factor catalyzes complex formation between the other factor and DNA, but does not itself remain on the complex. If both factors remain bound to the first gene, then addition of the rate-limiting factor with the second gene should be necessary and sufficient to increase transcription of the second gene. To test this, we formed stable complexes on the wild-type 5S gene with factors A, B, and C present at varying ratios and then tested the effects of further additions of the factors on transcription of the second gene. As shown in Fig. 4a, the addition of factor A with the second gene increased second gene transcription (lane 5 versus lane 4), whereas addition of either factor B or factor C was without effect (lanes 6 and 7). In an analogous experiment, the ratio of factor A to factor C was made 50 times greater (Fig. 4b); in this case the subsequent addition of factor C enhanced second gene transcription (lane 7 versus lane 4), whereas factors A or B alone had no effect (lanes 5 and 6). The observation that factors A and C can independently be made rate-limiting for second gene transcription suggests that each is bound in the complex on the first gene and that neither functions catalytically in promoting stable complex formation by the other. However, since the transcription reactions with these separated factors yield only about one transcript per gene, we can conclude that they are bound in a

stable preinitiation complex but not that they remain stably associated through many rounds of transcription.

Factor A forms a metastable complex. Although factors A and C bind to the 5*S* gene in a stable fashion (resistant to dissociation in the presence of a competing gene) only when both factors are present, the possibility exists that one or both may interact independently with the 5*S* gene to form a metastable complex. Such a metastable complex might be demonstrated if conditions could be found to rapidly convert it into a stable complex in the presence of a second gene. Therefore, we used a two-step protocol (Fig. 2) in which a previously incubated mixture (30°C) of the first gene and a limiting amount of the first factor (A or C) was added to an equilibrated mixture (30°C) containing an excess of the second stabilizing factor (C or A), factor B, RNA polymerase III, and the second gene. The success of this experiment is dependent on the rate of complex conversion by the stabilizing factor being greater than the rate of dissociation of the initial complex.

As shown in Fig. 5a, incubation of factor A with the first gene (wild type) followed by addition of the second gene (maxigene) with an excess of factor C resulted in preferential transcription of the first gene (lane 3). The reduction in the transcription of the second gene was nearly as complete as when both factors A and C were present in the first incubation with the primary gene (lane 2). This apparent interaction of factor A and the 5*S* gene was not observed in the experiment described above (data of Fig. 3) because (i) the ratio of factor C to factor A was eight times lower and (ii) the secondary gene and the transcription components were added to the reactions sequentially; this allowed a small but apparently sufficient amount of time for the complex to dissociate in the absence of factor C (thus establishing the equivalent of a three-step incubation-competition protocol as in the following experiment).

To further establish the metastable nature of the complex described immediately above, we used a three-step incubation-competition protocol (refer to Fig. 2) in which the secondary transcription factors were added 10 minutes after the addition of the secondary (maxigene) template, thereby allowing an extended period at 30°C for dissociation. As shown in Fig. 5a, under these conditions the presence of factor A alone in the initial incubation was insufficient to exclude transcription of the second gene; this resulted in equal transcription of the two genes (lane 6), as was observed when both templates were present in the first incubation (lane 4). Thus, the complex of the primary gene and factor A, which is trapped by simultaneous exposure to an excess of factor C and a second template, is not stable when incubated with a second template in the absence of factor C.

To detect interactions of factor C with the 5*S* gene in the absence of factor A (Fig. 5b), we used a rate-limiting amount of factor C (in the first incubation) and an excess of factor A (added later) in two-step (lanes 1 to 3) and three-step (lanes 4 to 6) incubation-competition experiments. In this case, prior incubation of the primary (wild-type) gene with factor C did not exclude transcription of the second template (maxigene) when the remaining factors (including A) were added together with (lane 3), or 10 minutes after (lane 6), the secondary gene. Thus, under conditions of rate-limiting C, we were unable to detect any metastable interaction of factor C and the 5*S* gene. Under conditions of limiting factor A (lane 5 in Fig. 5a) or factor C (lane 5 in Fig. 5b), the rigorous three-step protocol again demonstrates that both factors A and C are needed for stable complex formation.

Factor C is also necessary for tRNA and VA RNA transcription. When a mixture of 5*S* and tRNA genes or a mixture of 5*S* and VA RNA genes was incubated in an unfractionated extract (data not shown) or in a system reconstituted with separated factors, the tRNA gene (lane 3 in Fig. 6a) or the VA RNA gene (lane 3 in Fig. 6b) was transcribed to the exclusion of the 5*S* gene. These results indicate the presence of a common factor for which the tRNA or the VA RNA gene competes more effectively. To determine whether this component is one of those involved in stable complex formation on the 5*S* gene, we incubated the wild-type

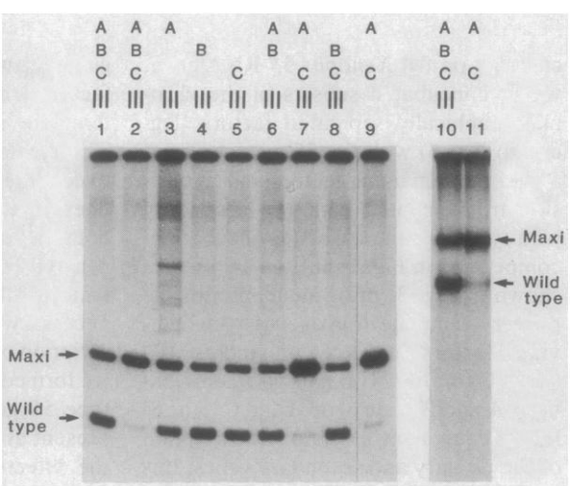

Fig. 3. Analysis of the human transcription factors that are necessary when incubated with a 5*S* RNA gene to exclude transcription from a second 5*S* gene. The autoradiograph shows the gel analysis of transcription reactions carried out in vitro with separated transcription factors. Initial incubation reactions contained the factors indicated above each lane and either 0.25 μg of the maxigene (p115/77) alone (lanes 2 to 9 and lane 11); or 0.25 μg each of the 5*S* maxigene and the wild-type 5*S* gene (pXBS1) (lanes 1 and 10). After the initial incubation, the reactions were cooled (4°C) and mixed sequentially with 0.25 μg of wild-type 5*S* RNA gene (lanes 2 to 9 and lane 11 only), any transcription factors (including RNA polymerase III) not present in the initial incubation, and nucleoside triphosphates. In this and subsequent experiments the first incubation was for 10 minutes at 30°C and transcription following nucleoside triphosphate addition was for 60 minutes at 30°C. The final reaction volume was 50 μl and contained 20 m*M* Hepes (*p*H 7.9), 70 m*M* KCl, 7 m*M* $MgCl_2$, 12 percent glycerol, 5 m*M* dithiothreitol, 600 μ*M* each of adenosine, uridine, and cytidine triphosphates, and 25 μ*M* [α-^{32}P]guanosine triphosphate (54 Ci/mmole). The initial incubation conditions were essentially those of the final incubation except for the absence of all nucleoside triphosphates. As precautionary measures the secondary incubation reactions also contained 0.02 μg of purified poly(adenosine diphosphate–ribose) polymerase to decrease random initiation by RNA polymerase III at nicks (*36*) and 10 units of placental ribonuclease inhibitor (Bolton Biologicals). After transcription was terminated the RNA's were purified (*11*) and fractionated by electrophoresis in 10 percent polyacrylamide gels containing 7*M* urea. The transcription factors A, B, and C were separated by chromatography of a HeLa cell extract on phosphocellulose (*9*). The 0.1*M* KCl phosphocellulose fraction was further purified on DEAE-cellulose (DE52) (*9*); a 0.25*M* KCl step was used as the standard factor A preparation. The 0.35*M* KCl phosphocellulose fraction was used as the standard factor B preparation. The 0.6*M* KCl phosphocellulose fraction was further purified by chromatography on DEAE-cellulose (*9*) and Bio-Gel A1.5M; the excluded fraction from the latter served as the standard factor C preparation. RNA polymerase III was isolated from HeLa cells (*9*). Unless otherwise noted, the experiments described in this and other figures contained 25 μg of factor A, 15 μg of factor B, 1 μg of factor C, and 50 to 100 units of RNA polymerase III (*37*). Factor B contained a substantial amount of RNA polymerase III activity, but factors A and C contained only trace amounts. Factor A contained a residual amount of factor B activity (about 2 percent of that in factor B). Otherwise the factors were not detectably cross contaminated. For the experiments in lanes 10 and 11, contaminating B activity was removed from factor A by further purification on DEAE-Sephadex (A25) and phosphocellulose (P11); the factor C used in these lanes was also further purified on DEAE-Sephadex (A25) and carboxy-methylcellulose (C25).

5S gene first with a mixture of all the factors and then with the tRNA or the VA RNA gene. In this case, the 5S gene was transcribed to the near exclusion of the tRNA gene (lane 4 in Fig. 6a) or the VA gene (lane 4 in Fig. 6b). Control experiments indicated that prior incubation with pBR322 did not significantly depress transcription of the secondary tRNA or VA I gene templates (compare lanes 1 and 2 in Fig. 6, a and b), thereby demonstrating the specificity of the competition for 5S genes and the stability of the factors during the first incubation. Thus, at least one factor that is stably bound in the 5S gene complex is also required for transcription of tRNA and VA I RNA genes.

To identify this common factor, we determined which isolated factor would relieve inhibition of tRNA or VA RNA gene transcription when added (with the tRNA or VA gene) in the competition assay. Data in Fig. 6 indicate that the inclusion of additional factor C restored tRNA or VA RNA synthesis (lanes 6 in Fig. 6, a and b), whereas additional factor B had no effect (lanes 5 in Fig. 6, a and b). Thus, a component of factor C involved in stable complex formation on 5S genes is also necessary for tRNA and VA gene transcription.

Complexes on the tRNA and VA RNA Genes

Factors B and C are required for a stable tRNA gene complex. As discussed above, the tRNA and VA RNA genes compete for a transcription factor required for 5S gene transcription. To establish whether the tRNA gene forms a stable complex with one or more factors, we used the three-step protocol with separated factors and the VA gene as the competing template. As shown in Fig. 7b, the presence of both factors B and C in the initial incubation with the tRNA gene was sufficient to preclude transcription of the second template (lane 2). Prior incubation of the first gene with either factor alone (lanes 3 and 4 in Fig. 7b) resulted in a transcription pattern indistinguishable from that observed when both genes were initially incubated together with the required factors (lane 1).

To determine whether one of the factors interacts independently in a less stable fashion with the tRNA gene, we used the two-step incubation-competition assay (Fig. 2) and conditions analogous to those used to demonstrate a metastable interaction of the 5S gene and factor A (see above). As shown in Fig.

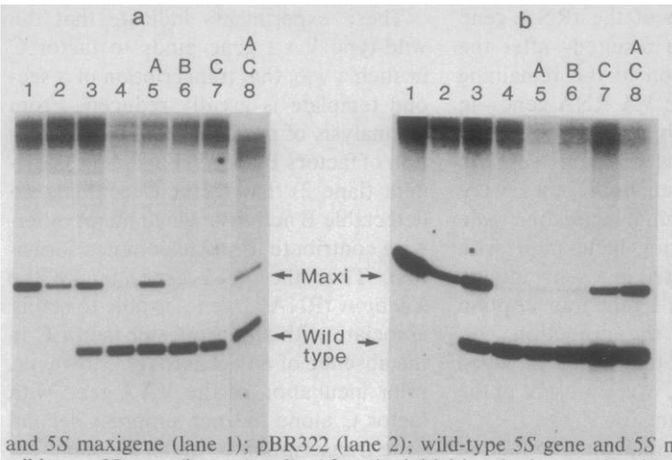

Fig. 4. Identification of components that are sequestered by the 5S gene and rendered rate-limiting for transcription of a second 5S gene. An autoradiography of transcription products separated by polyacrylamide gel electrophoresis is shown. In both (a) and (b) a mixture of factors A, B, and C was first incubated with 0.03 μg each of the following templates: pBR322 and 5S maxigene (lane 1); pBR322 (lane 2); wild-type 5S gene and 5S maxigene (lane 3); and wild-type 5S gene (lanes 4 to 8). After the initial incubation, nucleoside triphosphates and the additional transcription factors indicated above each lane were added with the following templates: none (lane 1); 5S maxigene (lane 2); none (lane 3); and 5S maxigene (lanes 4 to 8). In (a) the factor A, B, and C portions contained 3, 15, and 1 μg of protein, respectively. In (b), the factor A, B, and C portions contained 12.5, 15, and 0.08 μg of protein, respectively.

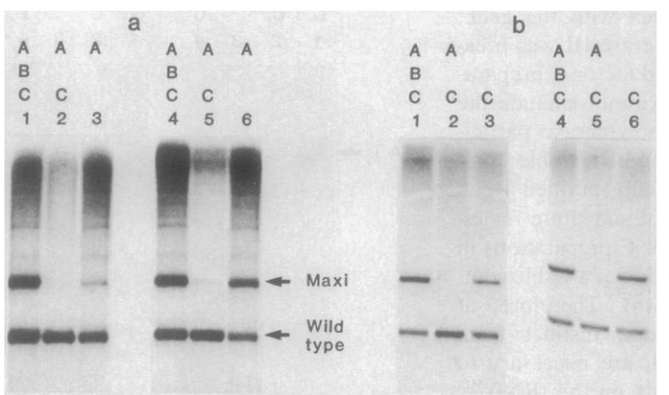

Fig. 5. Identification of the component that forms a metastable complex with the 5S gene. An autoradiograph of transcription products separated by polyacrylamide gel electrophoresis is shown. In lanes 1 and 4 of both (a) and (b), 0.03 μg each of the wild-type 5S gene and the 5S maxigene were first incubated with the factors indicated above each lane. In lanes 2, 3, 5, and 6, 0.03 μg of the wild-type 5S gene was first incubated with the factors indicated above each lane. The incubated reactions in lanes 1 to 3 were subsequently added (at 30°C) to equilibrated mixtures (at 30°C) containing nucleoside triphosphates, those transcription factors not present in the initial incubation, and either no additional DNA (lane 1) or 0.03 μg of 5S maxigene (lanes 2 and 3). The previously incubated reactions in lanes 4 to 6 were mixed (at 30°C) with either no additional DNA (lane 4) or 0.03 μg of 5S maxigene (lanes 5 and 6); after an additional 10 minutes of incubation at 30°C those transcription factors not present in the initial incubation were added along with the nucleoside triphosphates. In (a) the levels and proportions of factors A, B, and C were the same as in Fig. 4a whereas in (b) they were the same as in Fig. 4b.

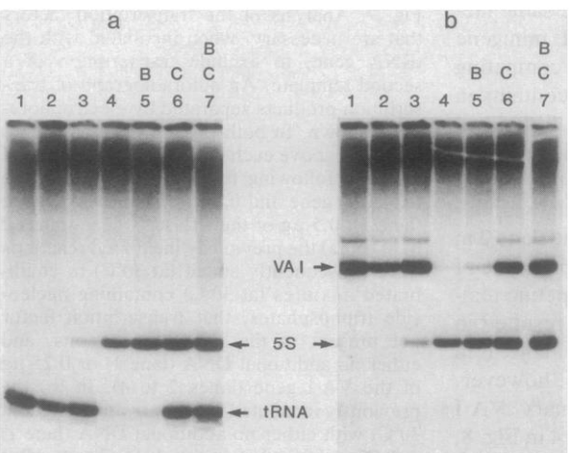

Fig. 6. Identification of a component that is sequestered in the 5S gene stable complex and necessary for tRNA and VA I transcription. An autoradiograph of transcription products separated by gel electrophoresis is shown. In (a), a mixture of factors A, B, and C was first incubated with 0.12 μg each of the following templates: pBR322 and tRNA$_1^{Met}$ (pX1tmet1) (lane 1); pBR322 (lane 2); wild-type 5S gene and tRNA$_1^{Met}$ gene (lane 3); wild-type 5S gene (lanes 4 to 7). After the initial incubation, nucleoside triphosphates and the additional transcription factors indicated above each lane were added along with the following templates: none (lane 1); tRNA$_1^{Met}$ gene (lane 2); none (lane 3); and tRNA$_1^{Met}$ gene (lanes 4 to 7). In (b) the additions were exactly the same except that the VA I gene (pVA) was used in place of the tRNA gene. In both (a) and (b), the amounts of factors A, B, and C used were as indicated in Fig. 4b.

7a, prior incubation of the tRNA gene with factor B alone resulted—after the simultaneous addition of the remaining components and the VA RNA gene—in transcription of both genes (lane 3); the relative amounts were equivalent to those observed when both genes were present during the initial incubation (lane 1). In contrast, prior incubation with factor C alone resulted in a substantially reduced level of VA I gene transcription (lane 4), although the reduction was somewhat less than that observed when both factors B and C were present in the initial incubation (lane 2).

The above results indicate that factors B and C are both necessary to form a stable transcription complex on the $tRNA_1^{Met}$ gene in such a way that transcription of a competing VA I gene is inhibited. Although factor C can interact with the tRNA gene, it cannot by itself form a stable complex with the gene. Because RNA polymerase III was present in the factor B and factor C preparations used here, we cannot exclude the possibility that the polymerase participated in the formation of a stable complex. However, partially purified RNA polymerase III cannot substitute for either factor B or factor C preparations in supporting the formation of a stable complex (data not shown). Therefore, at least two components, distinct from RNA polymerase III, are necessary to form a stable complex on the $tRNA_1^{Met}$ gene.

Factor C alone forms a stable complex on the VA I gene. To ascertain whether the VA I gene forms a stable complex with transcription factors, we used the three-step competition protocol. This protocol can specifically detect a stable complex and unmask a transient interaction. We used the wild-type VA I gene as the primary template and either the $tRNA_1^{Met}$ gene (which uses the same factors) or a homologous VA I minigene (pVA dl 1) as the secondary competing gene. The VA I minigene contains a small deletion but retains a functional promoter (*8*). Prior incubation of the primary (VA I) gene with factors B and C eliminated transcription of the secondary tRNA gene or VA minigene (lane 2 in Fig. 8, a and b), whereas the presence of both the primary and the competing template in the initial incubation resulted in equal levels of transcription (lane 1 in Fig. 8, a and b). Significantly, however, prior incubation of the primary VA I gene with factor C alone (lane 4 in Fig. 8, a and b), but not with factor B alone (lane 3 in Fig. 8, a and b), significantly depressed transcription of the second template.

These experiments indicate that the wild-type VA I gene binds to factor C in such a way that transcription of a second template is greatly reduced. From an analysis of possible cross contamination of factors B and C (Fig. 8c), it is evident (lane 2) that factor C contains no detectable B activity, which might otherwise contribute to stable complex formation. Thus the VA I gene, unlike the *Xenopus* $tRNA_1^{Met}$ gene, is able to stably associate with and sequester factor C in the absence of any B activity. However, prior incubation of the VA I gene with factor C alone did not suppress second gene transcription to quite the same extent as prior incubation with both factors B and C (compare lanes 2 and 4 in Fig. 8, a and b). Thus, although factor C alone binds tightly to the VA I gene, the presence of factor B apparently increases the stability of the factor C–VA I gene complex.

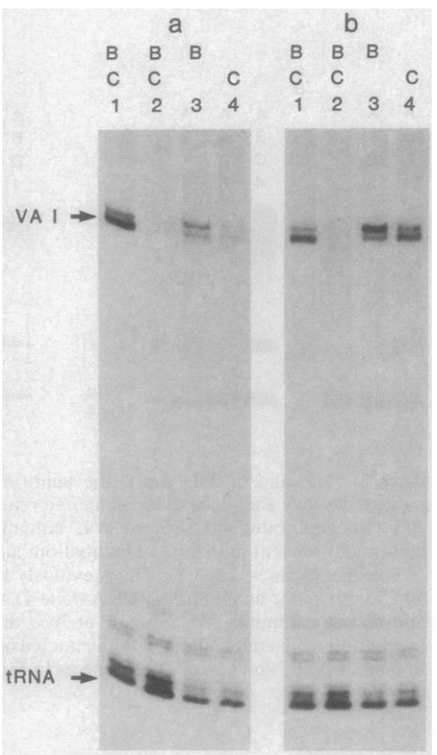

Fig. 7. Analysis of the transcription factors that are necessary, when incubated with the tRNA gene, to exclude transcription of a second template. An autoradiograph of transcription products separated by electrophoresis is shown. In both (a) and (b), the factors indicated above each lane were first incubated with the following templates: 0.5 μg of the $tRNA_1^{Met}$ gene and 0.25 μg of the VA I gene (lane 1); 0.5 μg of the $tRNA_1^{Met}$ gene (lanes 2 to 4). In (a) the previously incubated reactions were subsequently added (at 30°C) to equilibrated mixtures (at 30°C) containing nucleoside triphosphates, that transcription factor not present in the initial incubations, and either no additional DNA (lane 1) or 0.25 μg of the VA I gene (lanes 2 to 4). In (b) the previously incubated reactions were mixed (at 30°C) with either no additional DNA (lane 1) or 0.25 μg of the VA I gene (lanes 2 to 4); after an additional 10 minutes of incubation at 30°C, that transcription factor not present in the initial incubation was added along with the nucleoside triphosphates.

Factor B association with stable complexes during transcription. Factor B is not needed to form a stable complex on the 5*S* gene. However, this factor stabilizes the metastable association between factor C and the tRNA gene and increases the relative stability of the factor C–VA I gene complex. Therefore, we were prompted to examine whether factor B can stably associate with a stable complex and remain bound to the template during a transcription reaction. In the first experiment the VA I gene was incubated with factor C plus decreasing amounts of factor B, and the $tRNA_1^{Met}$ gene was separately incubated only with factor C. After 10 minutes, the two reactions were combined, mixed with RNA polymerase III and substrates, and incubated for an additional hour. If factor B remained bound to the factor C–VA I gene complex under conditions of rate-limiting factor B, no tRNA transcription would be expected. In the experiment shown in lanes 2 to 6 of Fig. 9a, total transcription was proportional to the amount of added factor B, an indication that factor B was present in rate-limiting amounts. Under these conditions, transcription of the tRNA gene (incubated only with factor C) is always observed. Therefore, within the limits of our assay, factor B does not appear to remain stably bound to the factor C–VA I gene complex during the 1-hour transcription reaction.

In a similar experiment, the tRNA gene was incubated with factor C and decreasing amounts of factor B, and the VA I gene was incubated with factor C alone. The reactions were then mixed and incubated with RNA polymerase III and nucleoside triphosphates. From the experiment shown in lanes 2 to 6 of Fig. 9b, it is evident that transcription of both genes occurs at all factor B concentrations. Thus, factor B does not appear to remain stably bound to the factor C–tRNA gene complex under transcription conditions. In similar experiments with 5*S* genes, we were not able, in the presence of a rate-limiting amount of factor B, to find evidence for a stable interaction between factor B and the complex formed by the 5*S* gene and factors A and C under transcription conditions (data not shown).

Although both genes in Fig. 9 were transcribed at all concentrations of factor B, the template initially incubated with both factors B and C was in all cases

preferentially transcribed in the subsequent incubation. Therefore, it is possible that (i) factor B did bind stably to the first template but cycled onto the other template during the transcription reaction, or (ii) the assay conditions did not allow complete binding of factor B during the initial incubation.

Site of Interaction of a Common Transcription Factor

Factor C interaction with the posterior half of the tRNA gene. Both the A and B sequence blocks are necessary components of the tRNA gene promoter. Prior incubation of the intact tRNA gene with factors B and C completely eliminated subsequent VA I gene transcription (lane 2 in Fig. 10a). Prior incubation of the factors with a subclone of the anterior region of the tRNA gene (containing the A block) did not reduce VA I transcription significantly more than prior incubation with pBR322 (compare lanes 3 and 5). However, prior incubation with a subclone of the posterior region (containing the B block) reduced VA I transcription to about one-fifth that observed after prior incubation with pBR322 (compare lanes 4 and 5). [The posterior region subclone directed transcription of two novel RNA species (lane 4), which are more apparent in lane 8 of Fig. 10b. These RNA's have not been mapped in detail, but both are derived specifically from the posterior region subclone, which is transcribed at about 2 percent of the efficiency of the intact tRNA gene.]

The posterior region of the tRNA gene contains the B sequence block and presumably inhibits VA I transcription through an interaction with one or more transcription factors. To clarify whether this region interacts with a common factor necessary for 5*S* gene transcription, a competition experiment was performed with the 5*S* gene. Prior incubation of the intact tRNA gene with factors B and C completely inhibited subsequent 5*S* RNA synthesis (lane 2 in Fig. 10b). Prior incubation of the posterior region of the tRNA gene with these factors (lane 4) depressed subsequent 5*S* RNA transcription to one-fourth the level observed after an initial incubation with either the subclone of the anterior region (lane 3) or pBR322 (lane 5). The depression of 5*S* RNA synthesis was relieved by additional factor C (lane 8), but not additional factor B (lane 7). Thus, prior incubation of the factors with the posterior region of the tRNA gene depresses 5*S* gene transcription because of the functional depletion of factor C. A subclone containing the A sequence block of the tRNA gene does not functionally sequester this factor more than does pBR322.

Factor C interactions near the B block of the VA RNA gene. A likely factor C recognition site in the posterior half of the tRNA gene is the B sequence block. Because of the relatively greater stability of the complex of factor C and the VA I gene, we examined whether factor C directly interacts with the B block homology in this gene. We took advantage of the presence of a single Bam HI recognition site located one nucleotide downstream from the B sequence block in the VA I gene (see Fig. 11), reasoning that a stable factor C interaction with the B sequence block might protect the adjacent Bam HI site from cleavage. For controls we monitored the protection of the Sal I site at the border of the Ad2 insert in pVA and the Bam HI and Sal I sites in pBR322 in the presence of factor C.

In the experiment shown in Fig. 11, pBR322 and pVA were mixed after they had been cleaved and end-labeled at the single Eco RI site present in each plasmid. These DNA's were incubated first with factor C for 10 minutes and then with Bam HI for an additional 2.5, 5, 10, and 20 minutes (lanes 1 to 4). Whereas the pBR322 Bam HI site was completely cleaved after 5 minutes in the presence of factor C (lane 2), 90 percent of the pVA Bam HI sites remained resistant to

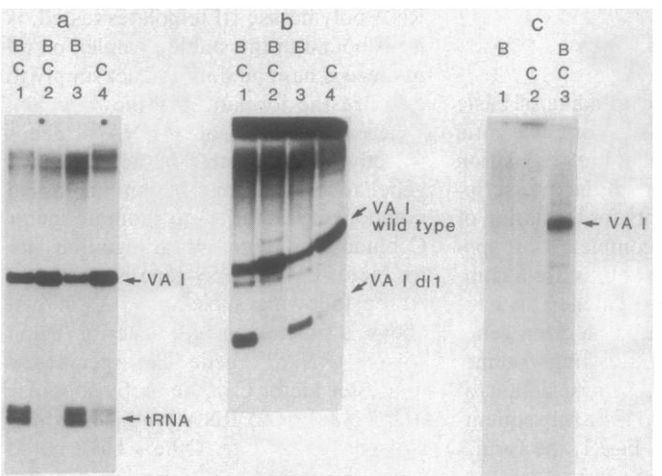

Fig. 8. Analysis of the transcription factors that are necessary when incubated with the VA I gene to exclude transcription of a second template. In (a) the factors indicated above each lane were first incubated with either 0.25 μg each of the VA I and the $tRNA_1^{Met}$ genes (lane 1) or 0.25 μg of the VA I gene alone (lanes 2 to 4). The initially incubated reactions were mixed (at 30°C) with either no additional DNA (lane 1) or with 0.25 μg of the $tRNA_1^{Met}$ gene (lanes 2 to 4); after an additional 10 minutes of incubation at 30°C, that transcription factor not present in the initial incubation was added along with the nucleoside triphosphates. In (b) the additions were identical except that 0.75 μg of the VA I deletion mutant (pVA I dl 1) was added in place of the $tRNA_1^{Met}$ gene. In (c) the VA I gene (0.25 μg) was incubated under transcription conditions in the presence of 100 units of RNA polymerase III and the factors indicated above each lane.

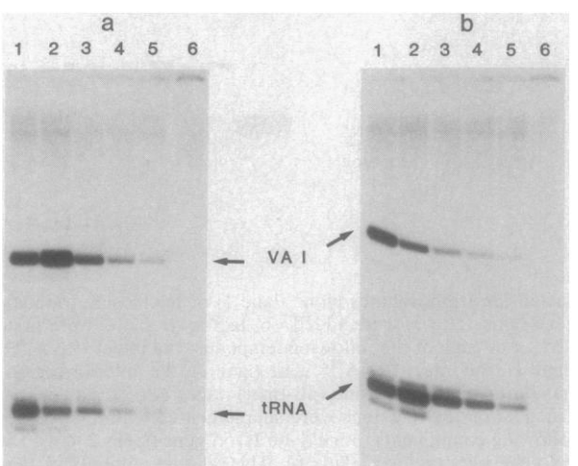

Fig. 9. Does factor B remain sequestered in a stable complex? An autoradiograph of transcription products separated by polyacrylamide gel electrophoresis is shown. (a) In one set of reactions 0.3 μg of the VA I gene was incubated for 10 minutes with a constant amount of factor C (0.4 μg) plus the following amounts of factor B: 0.75 μg (lane 1), 1.5 μg (lane 2), 0.38 μg (lane 3), 0.19 μg (lane 4), 0.09 μg (lane 5), or none (lane 6). In a second set of reactions, 0.3 μg of the $tRNA_1^{Met}$ gene was incubated for 10 minutes with 0.4 μg of factor C plus either 0.75 μg of factor B (lane 1) or with factor C alone (lanes 2 to 6). The corresponding reactions were then mixed together, supplemented with RNA polymerase III and nucleoside triphosphates, and incubated for an additional hour at 30°C. (b) The same experimental regime as described in (a) was used; however, the tRNA gene was initially incubated with factor C plus decreasing amounts of factor B, and the VA I gene was initially incubated with factors B and C (lane 1) or with factor C alone (lanes 2 to 6).

cleavage after 20 minutes of incubation with the enzyme (lane 4). Similarly, the labeled DNA's were incubated with factor C, followed by digestion with Sal I for 2.5, 5, 10, and 20 minutes (lanes 5 to 8). The Sal I site in both plasmids was equally accessible to this restriction enzyme in the presence of factor C. Protection of the Bam HI site within the VA I gene was not observed after incubation of the DNA with bovine serum albumin or with heat-treated factor C (80°C for 10 minutes; data not shown). Therefore, the VA I Bam HI site is specifically and stably protected by a heat-labile component present in factor C. Protection of the Bam HI site by factor C could be due either to a steric blockade of the site or to an alteration of the sequence topology (discussed below).

Conclusions and Implications

To further our understanding of basic transcription mechanisms for eukaryotic genes, and ultimately the regulation thereof, we investigated the transcription factor requirements for formation of stable (preinitiation) complexes on specific genes. These complexes are a common feature of class III genes and are inferred from the ability of a given gene, after its incubation with limiting amounts of factors, to preclude transcription of competing templates added subsequently. As summarized in Fig. 1, the formation of such complexes requires factors A and C for 5*S* genes, factors B and C for a tRNA gene, and only factor C for the adenovirus VA I RNA gene. These factors are presumably the key factors involved in initial promoter recognition but represent only a subset of those required overall for transcription. It should be emphasized that these experiments, because of the assay employed, do not exclude the possibility that the other factors might stably associate with these complexes. It is, in fact, likely that such complexes serve as recognition sites for these other essential components (factor B and RNA polymerase III), which could cycle during the reaction (see below).

Factor C is a pivotal component of stable complexes. Factor C, which is needed to form a stable complex on all RNA polymerase III templates tested, is itself bound in the stable complex on all of these genes and does not act simply to catalyze the formation of the complex. Whereas the VA I and $tRNA_1^{Met}$ genes directly interact with this factor, the 5*S* gene appears to require an additional component (factor A) to promote factor C binding. A potential recognition site for factor C on the 5*S* gene is discussed in the following subsection. As shown above, a subclone of the posterior region of the $tRNA_1^{Met}$ gene can specifically sequester factor C and thereby suppress either VA I or 5*S* RNA synthesis from a secondary template. Others have noted that the 3′ half of the $tRNA_1^{Met}$ gene, containing the B block (Fig. 1) specifically depresses transcription of the intact gene in an unfractionated oocyte extract (*22*), and the formation of a stable complex on a *Drosophila* $tRNA^{Arg}$ gene in crude cellular extracts requires sequences in the B block (*23*). It therefore seems probable that binding of factor C to the posterior region of the $tRNA_1^{Met}$ gene is directly or indirectly dependent on interaction with the B sequence block. In support of this hypothesis, we demonstrated that factor C specifically protects from endonucleolytic cleavage the Bam HI site located just 3′ of the B sequence block in the VA I gene. The prolonged protection of this site (for at least 20 minutes) in the presence of a vast excess of the restriction enzyme strongly indicates a stable interaction of DNA and protein and is consistent with the stable interaction of the VA I gene with factor C observed in the incubation-competition assay. Protection of this site could be due either to a steric blockade or to an alteration of the topology of the DNA in this region. The B sequence block in the VA I gene has the potential to form a stem-loop structure (see below) that would include two nucleotides of the Bam HI recognition site and therefore preclude cleavage. Using deoxyribonuclease I protection experiments, we recently demonstrated that factor C interacts over the entire B sequence block of the VA I gene (*12*). Similarly, a yeast homogenate contains a factor that interacts with the B block of the VA I gene and several tRNA genes (*24*).

An interaction of human factor C with the $tRNA_1^{Met}$ gene in the absence of other factors has not yet been demonstrated directly (for example, by deoxyribonuclease I protection experiments). However, the presence of such an interaction has been inferred from two-step incubation-competition experiments (Fig. 7) in which the stabilizing B factor (added in excess with the second template) is apparently able to transform an otherwise unstable complex into a stable complex (prior to dissociation of the former). The unstable interaction of factor C with the $tRNA_1^{Met}$ gene is not simply a consequence of the use of heterologous components, since an unstable interaction of the human $tRNA^{Met}$ gene with factor C (in the absence of factor B) has also been observed (*25*).

Although factor C alone binds tightly to the VA I gene, the stability of the factor C–VA I complex is apparently increased in the presence of factor B. Therefore, it seems likely that the VA I and $tRNA^{Met}$ genes interact in a qualita-

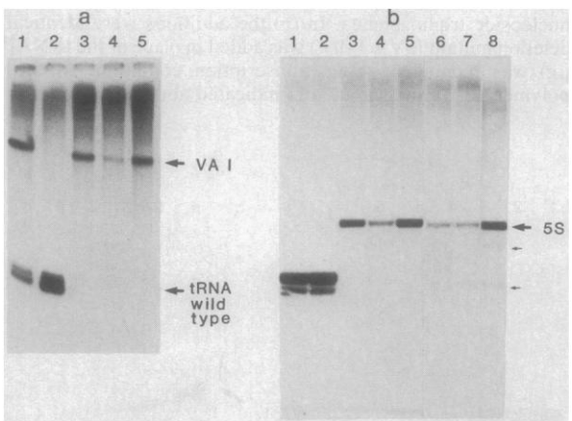

Fig. 10. Competition for transcription factors by the anterior and posterior regions of the $tRNA_1^{Met}$ gene. An autoradiograph of transcription products separated by polyacrylamide gel electrophoresis is shown. (a) Factors B and C were first incubated for 10 minutes with 0.25 μg of the following templates: the intact $tRNA_1^{Met}$ gene and the VA I gene (lane 1), the intact $tRNA_1^{Met}$ gene (lane 2), a subclone of the anterior region of the $tRNA_1^{Met}$ gene (pA-tmet) (lane 3), a subclone of the posterior region of the $tRNA_1^{Met}$ (pB-tmet) (lane 4), or pBR322 (lane 5). After the initial incubation, nucleoside triphosphates alone (lane 1) or nucleoside triphosphates and 0.25 μg of the VA I gene (lanes 2 to 5) were added. (b) factors B and C were first incubated for 10 minutes with 0.025 μg of each of the following templates: the intact $tRNA_1^{Met}$ gene and the wild-type 5*S* gene (lane 1), the intact $tRNA_1^{Met}$ gene (lane 2), the anterior region subclone (pA-tmet) (lane 3), the posterior region subclone (pB-tmet) (lanes 4, 6, 7, and 8), or pBR322 (lane 5). After the initial incubation the reactions were supplemented with nucleoside triphosphates, factor A, and the following components: none (lane 1), 5*S* gene (lanes 2 to 6), 5*S* gene plus factor B (lane 7), or 5*S* gene plus factor C (lane 8). The relative amounts of the transcription factors used in (b) were the same as in Fig. 4b (that is, the competition was performed under conditions of limiting factor C). The small arrows designate the position of RNA's derived from the posterior region subclone (pB-tmet). The anterior and posterior region subclones of the *Xenopus laevis* $tRNA_1^{Met}$ gene were constructed as described (*22*) except that the anterior (upstream from residue 30) and the posterior (downstream from residue 31) fragments were individually blunt-end ligated into the Hind III site of pBR322.

tively similar manner with the transcription factors, but that they are distinguished by quantitative differences in relative affinities for factor C. If tRNA (or VA RNA) promoter function involves the factor-dependent formation of a stem-loop structure in the B block region, as suggested by Hall *et al.* (*5*), the more stable interaction of factor C with the VA gene could be due to a greater stability of the VA gene stem-loop [a stem of six GC pairs (G, guanine; C, cytosine) and a loop of six bases versus the tRNA gene stem-loop [a stem of three GC and two AT (A, adenine; T, thymine) pairs and a loop of seven bases]. Alternatively, the relative stabilities of the factor C–DNA complexes could result from sequence differences in this region (*7*).

The 5S gene complex involves both gene-specific and common factors. The human factor A interacts with the 5*S* gene in the absence of other factors, as demonstrated by the two-step incubation-competition assay and as anticipated from previous studies of the *Xenopus* factor A (*10, 26, 27*). However, as suggested for the *Xenopus* factor A complex (*16*), the human factor A–5*S* gene complex is unstable and readily dissociates in the presence of another 5*S* gene. We demonstrated that this unstable association is transformed into a stable complex in the presence of factor C, which appears to remain associated with this complex. Thus far, we have not been able to demonstrate, with the two-step incubation-competition assay, any independent interactions of factor C with the 5*S* gene, even though this factor forms complexes with the VA gene (tight) and the tRNA gene (weak) under the same conditions.

The foregoing observations suggest that the order of interaction of factors with the 5*S* gene is factor A followed by factor C. They also raise questions about the factor C recognition site and possible alterations of the factor A interactions in the presence of factor C. Apart from the fact that the factor A interaction appears to involve the entire 5*S* promoter sequence (Fig. 1), there is the additional observation that the factor C interaction site within the VA and tRNA gene involves a region (the B block) whose consensus sequence has not been clearly identified in the 5*S* promoter [(*6*); see below]. While an A block consensus sequence is common to the class III genes (*6*) and is a potential site of interaction for the common factor C, the failure to detect (in the competition assay) an interaction of factor C with a gene fragment containing this region makes it more likely that this is a recognition site for other common components, such as factor B or RNA polymerase III. These considerations raise the possibility that interaction of factor A with the promoter induces or establishes a factor C recognition site consisting either of DNA contacts or protein (factor A) contacts, or a combination of both. It may be significant that the interaction of the *Xenopus* factor A with the 5*S* gene induces three sites of deoxyribonuclease I hypersensitivity on the noncoding strand of the gene (*10, 27*), the most prominent of which is in a region with marked homology to the B sequence block of the tRNA promoter (*8*). It is conceivable that the factor A interaction increases the affinity of this site for factor C (as well as for deoxyribonuclease I). Deoxyribonuclease I protection studies should determine whether factor C requires a factor A "adaptor" function to recognize the promoter region of the 5*S* gene or whether the factor A (or factor C) interactions in binary (metastable) complexes are different from those in ternary (stable) complexes.

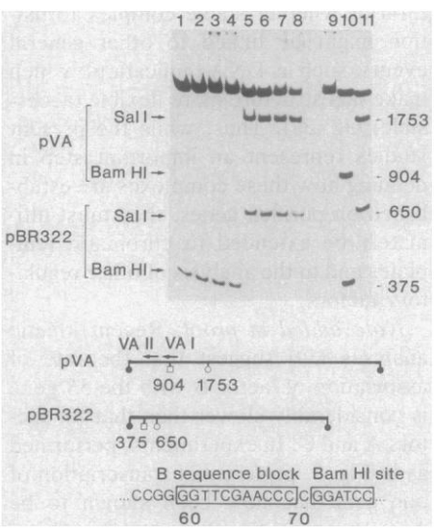

Fig. 11. Restriction site protection of a sequence adjacent to the B block in the VA I gene by factor C. An autoradiography of end-labeled DNA fragments separated by gel electrophoresis is shown. 2.5 ng each of the VA I plasmid (pVA) and pBR322, end-labeled at the Eco RI site, were mixed together with 0.3 μg of factor C. After 10 minutes of incubation at 30°C, 10 units of Bam HI (lanes 1 to 4) or 10 units of Sal I (lanes 5 to 8) were added, and the reactions were incubated at 30°C for another 2.5 minutes (lanes 1 and 5), 5 minutes (lanes 2 and 6), 10 minutes (lane 3 and 7), or 20 minutes (lanes 4 and 8). The end-labeled plasmids were incubated in the absence of factor C for 20 minutes at 30°C with no restriction enzyme (lane 9), 10 units of Bam HI (lane 10), or 10 units of Sal I (lane 11). The restriction maps of pVA and pBR322, both linearized at the Eco RI site, are shown: (●) an Eco RI site, (□) a Bam HI site; and (○) a Sal I site. The sequence of the noncoding strand of the VA I gene, adjacent to the B sequence block, is displayed; numbers below the sequence indicate the nucleotide distance downstream from the 5′ end of VA I RNA.

The transformation of a metastable complex of factor A and the 5*S* gene into a stable complex is reminiscent of the transition of a bacterial RNA polymerase "closed" promoter complex into a stable "open" (preinitiation) complex (*28*). Although it is unclear whether factors A and C induce the equivalent of an open complex on the 5*S* gene, there are several relevant observations. (i) The *Xenopus* factor A alone effects a small change in helix rotation (equivalent to an unwinding of 2 to 4 base pairs) (*29*). (ii) The *Xenopus* factor A makes contacts with phosphate and guanine residues primarily on the noncoding strand of the 5*S* gene promoter (*27*); this leads to the suggestion that factor A might transiently shift to the noncoding strand during transcription. (iii) Preliminary studies (*30*) indicate that the *Xenopus* factor A, in the presence of other factors from an oocyte extract, can bind specifically to a single-stranded M13 clone containing the noncoding strand of the 5*S* gene. It is tempting to speculate, therefore, that factor C may enhance the interaction of factor A with the noncoding strand of the 5*S* gene and thereby induce a stable "open" complex on the gene.

Sequence of factor interactions. On the basis of our analyses of factor requirements for formation of metastable as opposed to stable complexes, it appears that the order of factor interactions for the 5*S* gene is factor A, factor C, and factor B or RNA polymerase III, whereas that for tRNA and VA RNA genes is factor C, factor B, and RNA polymerase III.

Although we have no information on the role of factor B in 5*S* gene transcription, it appears to stabilize the factor C–tRNA gene complex and, to a much lesser extent, the factor C–VA gene complex. We have attempted to determine whether factor B simply catalyzes the stable association of factor C or is itself bound into a stable complex on these templates. Under our conditions of analysis, factor B does not appear to remain stably bound to the VA I and tRNA genes in the presence of factor C or to the 5*S* gene in the presence of factors A and C, respectively. Although it seems, from the assay employed, that factor B exchanges between templates during transcription in vitro, we cannot rule out the possibility that our incubation conditions do not permit the assembly of factor B into a stable complex that might otherwise exist in vivo (but see

note added in proof). In fact, the first study suggesting the presence of stable complexes within natural templates (*17*) indicated that isolated chromatin is associated with all the components (presumably including factors A, B, and C) necessary to promote transcription of the endogenous 5*S* genes by purified RNA polymerase III. We have also found that recombinant SV40 minichromosomes containing tRNA genes can be isolated in association with transcription factors B and C, but not RNA polymerase III (*25*). Thus, it is conceivable that factor B associates with the other factors in a preinitiation complex, which can be isolated with cellular and viral chromatin but which cycles during the transcription reaction. These chromatin studies, along with our demonstration that stable complexes can form on class III genes in the absence of RNA polymerase III, suggest that the final recognition event before transcription involves the RNA polymerase and protein-DNA contacts in the preinitiation complex.

Implications for gene regulation. Since the class III genes thus far tested share at least three common transcription factors (factors B, C, and RNA polymerase III), their relative activities during physiological transitions in which one or more factors become rate-limiting may be in part determined by their relative affinities for individual factors. The remarkable stability of the stable complexes also provides a means for maintaining the transcription potential of one set of genes to the exclusion of another, potentially competing, set (*16*) and emphasizes the special role of those factors involved in complex formation. One example of a situation in which these considerations may be relevant is the lytic infection of human cells by adenovirus 2. This process occurs with a high level of replication (many thousandfold) and transcription of the viral VA RNA genes (which account for the vast majority of class III transcripts). Although it is not yet established that any of the factors (particularly factor C) actually becomes rate-limiting during infection, the level of 5*S* synthesis remains unaltered, while that of tRNA synthesis is dramatically reduced (*31*). Apart from the fact that cellular factors might be modified during infection to accommodate the establishment of viral transcription complexes, it is also possible that the VA I gene may have evolved to bind factor C more tightly in the absence of other factors so as to compete more effectively for limiting amounts of this factor (free or bound) within the cell.

A second example concerns the differentially expressed classes of 5*S* RNA genes in *Xenopus* (*16*); these present a somewhat special case since they are subject to regulation by both common and gene-specific (factor A) components. Exactly how these genes are differentially regulated is unclear, but Brown and his colleagues (*16, 32*) have demonstrated that this reflects the selective establishment and maintenance of stable complexes on one class of genes in somatic cells that contain limiting amounts of factor A (*14*). These investigators have also described the general implication of stable complexes for the maintenance and propagation of the activated state of a gene.

A key issue is how these complexes are established in the cell within the context of the natural chromatin structures and, in particular, whether they can be established on a "static" structure (under the influence of other factors) or whether stable complex formation might be linked to other general events (such as DNA replication), which make this structure more flexible (accessible) (*32, 33*). Thus, while the present studies represent an important step in defining how these complexes are established on purified genes, they must ultimately be extended to chromatin templates and to the analysis of other regulatory factors.

Note added in proof: Recent kinetic analyses (*30*) suggest that the rate of association of factor B with the 5*S* gene is considerably slower than that for factors A and C. In experiments, performed as in Fig. 9, preferential transcription of one gene has now been shown to be directly proportional to the length of time this gene was initially incubated with factor B. This observation suggests that factor B is sequestered in a stable complex on class III genes only after an extended incubation (40 minutes).

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20. Throughout this study we have used human transcription factors in conjunction with heterologous genes (the *Xenopus* 5*S* RNA and $tRNA_i^{Met}$ genes), as well as the homologous VA I gene in human adenovirus 2. The conclusions based on the studies with amphibian genes are justified on the basis of the following: (i) the sequences of the human and amphibian $tRNA_i^{Met}$ species, and therefore the internal control sequences, are identical; (ii) the human 5*S* RNA and the *Xenopus* somatic 5*S* RNA species are 94 percent homologous overall and differ by only one base pair in the internal control region consisting of about 50 base pairs.
21. The recombinant DNA plasmids containing the *Xenopus borealis* somatic type 5*S* gene (pXBS1) and the 5*S* maxigene (p115/77) were obtained from D. Brown and his colleagues [R. C. Peterson, J. L. Doering, D. D. Brown, *Cell* **20**, 131 (1980); D. F. Bogenhagen and D. D. Brown, *ibid.* **24**, 261 (1981)]. The plasmids containing the adenovirus 2 wild-type VA I gene (pVA) and minigene (pVA dl 1) were obtained from T. Shenk (*8*). The construction of the plasmid containing the *Xenopus laevis* $tRNA_i^{Met}$ gene (pX1tmet1) is outlined in (*10*). B. Shastry of our laboratory provided us with plasmids containing the anterior (pA-tmet) and posterior (pB-tmet) regions of the $tRNA_i^{Met}$ gene.
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38. We thank M. Birnstiel, D. Brown, T. Shenk, B. Shastry, and M. Zasloff for recombinant DNA plasmids, and M. Sawadogo, J. Segall, and E. Slattery for preparations of factor C, RNA polymerase III, and poly(ADP-ribose) polymerase, respectively. A.B.L. thanks H. Sive and N. Heintz for advice and encouragement during the course of this work. Supported by research grants CA 24223 and CA 24891 from the National Cancer Institute and by grant NP-284 from the American Cancer Society. P.L.M. was supported by Medical Scientist training grant GM07200 from the National Institutes of Health.