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Novobiocin inhibits interactions required for yeast TFIIIB sequestration during stable transcription complex formation *in vitro*

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ABSTRACT

Novobiocin concentrations normally used to inhibit a putative eukaryotic DNA gyrase have been found to inhibit transcription of a yeast 5S rRNA gene using an in vitro yeast transcription system. Purified RNA polymerase III and three yeast transcription factors (chromatographically separated, partially purified and free of any detectable gyrase activity) were used. Novobiocin prevents specific transcription if added to the in vitro system immediately prior to the addition of transcription factors and RNA polymerase. If a stable transcription factor complex is allowed to form prior to the addition of novobiocin, concentrations of novobiocin as high as 1000 µg/ml have no effect on in vitro transcription. Transcription factors TFIIIA and TFIIIC are able to be stably sequestered onto 5SrDNA-cellulose, but factor TFIIIB is not able to associate with the 5SrDNA-TFIIIA-TFIIIC complex in the presence of novobiocin. Although novobiocin is able to precipitate other basic proteins, it does not appear to precipitate any of these class III gene transcription factors, but instead appears to act by disrupting specific factor-factor interactions.

INTRODUCTION

We have been interested in developing an in vitro system for the assembly of transcriptionally active chromatin and have chosen the yeast 5S rRNA gene as our model system. In this regard we have been pursuing the purification of the yeast class III transcription factors for use within the context of a purified chromatin assembly system. Several reports have appeared in the literature recently which suggest that an ATP-dependent topoisomerase II (DNA gyrase) is responsible for the assembly and maintenance of transcriptionally active chromatin in eukaryotes (1,2,3). An important part of the evidence for such an activity is that novobiocin (an inhibitor of bacterial DNA gyrase) prevents chromatin assembly and transcription of a Xenopus 5S rRNA gene in Xenopus oocytes and oocyte extacts (1,2,3). In bacterial systems, novobiocin is a competitive inhibitor of ATP binding to gyrase. The concentrations of novobiocin needed for this inhibition are in the range of 20-60 µg/ml (4,5). The amount of novobiocin used to inhibit the putative gyrase in eukaryotic systems is greater than 200 µg/ml (1,2,3,6). The more recent discovery that these higher concentrations of novobiocin are able to precipitate histones (7)

prompted us to question the usefulness of novobiocin for the study of transcription reactions. While these studies were in progress, Gottesfeld reported that intermediate concentrations of novobiocin (100-200 µg/ml) created a lag in stable transcription complex formation in HeLa cell extracts while the more specific inhibitor of topoisomerase II (VM-26) as well as antibodies to topoisomerase I and topoisomerase II had no effect on 5S rRNA gene transcription in these same extracts (6). Here we present data which suggest that novobiocin disrupts class III gene transcription by prohibiting the association of transcription factor TFIIIB with the other factors in the manner required for stable transcription complex formation.

MATERIALS AND METHODS

Recombinant Plasmid DNAs

All transcription assays using soluble DNA were with the plasmid pUC5S, a subclone of pSc5S (8). pUC5S consists of a 400bp TaqI fragment of *S. cerevisiae* DNA containing the 5S rRNA gene inserted into pUC9 at the AccI restriction site. DNA-cellulose used in some of the experiments was made using pSc5S according to the method reported by Setzer and Brown (9) and was kindly provided by M. Parsons. A 1:1 (v/v) suspension of the 5SrDNA-cellulose in buffer (BCM/100, see below) was prepared. The amount of suspended material needed for optimal transcription was based on its titration with the amount of each transcription factor used for transcription of soluble DNA templates.

Transcription Factors and RNA Polymerase III

Buffers: Buffer A = 20 mM Hepes (pH 7.9), 0.2 mM EDTA, 10 mM 2-mercaptoethanol, 0.1 mM PMSF, 1.0 mM benzamidine. Buffer C = Buffer A plus 20% (v/v) glycerol. BCM/0 = Buffer C plus 10 mM $MgCl_2$. BCM/100 = Buffer C plus 10 mM $MgCl_2$ and 100 mM NaCl. BCM/500 = Buffer C plus 10 mM $MgCl_2$ and 500 mM NaCl. Yeast transcription factors were prepared primarily by multistep column chromatography as previously reported (10,11). For the preparation of TFIIIA, yeast whole cell extracts were applied to a BioRex70 column. After washing with BCM/100, all three factor activities were eluted with Buffer C containing 700 mM NaCl (BC/700). The protein pool was concentrated by precipitation with ammomium sulfate (0.35 g/ml) and then dialyzed against BCM/500. This material was then applied to a Sephacryl S300 column and the protein fractions containing purified factor IIIA activity were pooled. For the preparation of TFIIIB, yeast whole cell extracts were applied to a Heparin-Sepharose column and washed with BC/100 and then Buffer C containing 250 mM NaCl (BC/250). The column was then eluted with BC/700. The protein fractions containing RNA polymerase III, TFIIIA, TFIIIB, and TFIIIC were pooled and concentrated by precipitation with ammonium sulfate (0.42 g/ml) and dialyzed against BCM/500.

This material was then applied to 10-30% (v/v) glycerol gradients and centrifuged for 20-24 hours at 40,000 rpm in a Beckman SW41 rotor at 4°C. Fractions containing factor TFIIIB activity were pooled and dialyzed against BCM/100. TFIIIC was prepared from yeast whole cell extracts by Phosphocellulose and DEAE chromatography as outlined previously (11). Each factor preparation was assayed for the presence or absence of the other factors. Although these are only partially purified protein fractions, we refer to them as factors. Each factor preparation was titrated to yield optimal transcription at a DNA concentration of 12.5 µg/ml. RNA polymerase III was purified by the method of Ruet et al. (12).

Transcription Assays

Specific transcription assays were performed as previously described (10,11) in a final volume of 50 µl containing template DNA (0.625 µg pUC5S DNA or 20 µl of a 1:1 suspension of 5SrDNA-cellulose containing approximately 2 µg DNA), 5 µl TFIIIA (0.33 mg/ml), 5-10 µl each of TFIIIB (20 mg/ml) and TFIIIC (4.4 mg/ml), 100 units RNA polymerase III (1 unit = 1 pmole UMP incorporated into RNA in 20 minutes at 30°C using calf thymus DNA as the template), 600 µM each ATP, CTP and UTP, 50 µM GTP and 10 µCi α-^{32}P-GTP (800 Ci/mmol, New England Nuclear). Preincubations were carried out in volumes convenient to the later addition of the appropriate factors, RNA polymerase III and nucleoside triphosphates (NTPs). In all cases, the buffer conditions were adjusted to maintain final concentrations of 125 mM NaCl and 8 mM $MgCl_2$. All incubations were at 22°C for 30-40 minutes unless otherwise indicated. Transcription reactions were stopped with an equal volume of buffer containing 0.4% SDS, 100 mM sodium acetate, and E. coli tRNA (1 mg/ml) as carrier. The samples were treated with proteinase K (0.2 mg/ml) for 1 hour at 37°C, extracted with equal volumes of phenol/chloroform (1:1) then chloroform/isoamyl alcohol (24:1) and precipitated with 2 volumes ethanol after being made 2 M in ammonium acetate. The RNA precipitates were dried under vacuum and dissolved in 15 µl sample buffer (50% formamide, 0.2% SDS, 0.1% bromophenol blue and xylene cyanol). The ^{32}P-labeled transcripts were resolved on 12% polyacrylamide gels in TBE buffer (90 mM Tris, 90 mM Boric Acid, 1 mM EDTA, pH 8.3) and visualized by exposure to X-ray film (Kodak XAR or BB5) for 12-16 hours at -70°C with an intensifying screen. Quantitation of transcription was performed by excising the portion of the dried gel containing the transcript, placing the gel slice in scintillation fluid (ReadiSolv MP, Beckman) and determining the ^{32}P-cpm by liquid scintillation counting. We generally obtain the incorporation of 4800 cpm/µg DNA/30 min (0.1 transcripts/gene/hr) in a normal transcription assay.

Novobiocin

Novobiocin (Sigma) was dissolved in water at a stock concentration of 10

mg/ml and stored in small aliquots at -20°C. Due to the possible breakdown of novobiocin by prolonged exposure to light, thawed aliquots were not re-used.

RESULTS

Novobiocin Inhibits 5S rRNA Gene Transcription In Vitro

The yeast 5S rRNA gene is effectively transcribed *in vitro* by yeast whole cell extracts (10). Purified yeast RNA polymerase III will specifically transcribe class III genes (tRNA, 5S rRNA, and certain viral genes) *in vitro* when specific protein factors are added. We and others (8,10,11,13) have shown that, like their mammalian and invertebrate counterparts, all yeast tRNA genes identified require two transcription factors (TFIIIB and TFIIIC) for their transcription by RNA polymerase III. Transcription of the yeast 5S rRNA gene requires the addition of a third factor, TFIIIA. We have chromatographically separated and partially purified these three transcription factors (see Ref. 11 and Materials and Methods) and have utilized them in our studies of the effects of novobiocin on class III gene transcription *in vitro*. In the experiment shown in Figure 1, increasing concentrations of novobiocin were added to the template DNA prior to the addition of transcription factors, polymerase and nucleoside triphosphates (NTPs). An autoradiogram of the ^{32}P-labeled transcripts produced in this experiment is shown. Two specific transcripts are often obtained *in vitro* (13) and are due to transcription termination at two specific sites (runs of T's) or due to termination at one site followed by some degree of processing during the reaction. Quantitation of the results shown in Figure 1 indicates that a novobiocin concentration of 250 µg/ml inhibits 5S rRNA gene transcription approximately 50%. Concentrations of 500 and 1000 µg/ml inhibit transcription nearly 100%. These findings are in agreement with results recently reported for 5S rDNA transcription in HeLa cell extracts (6). As also reported for this mammalian system, novobiocin does not interfere with the DNA in any manner tested. It does not intercalate into the DNA to cause any topological changes nor does it precipitate plasmid DNA (14).

Preformed Transcription Complexes are Resistant to Novobiocin

Segall (13) has recently shown that yeast transcription factors are able to to be stably sequestered onto 5S rDNA within 12 minutes after addition of the factors to the template. Studies with transcription factors from HeLa and *Xenopus* systems suggest that factor-DNA complexes remain stable through multiple rounds of transcription while RNA polymerase III dissociates and rebinds to reinitiate transcription (9,15,16,17). In the experiment presented above, novobiocin was added to the DNA prior to the addition of transcription factors. Hence, it was not possible to discern whether novobiocin was

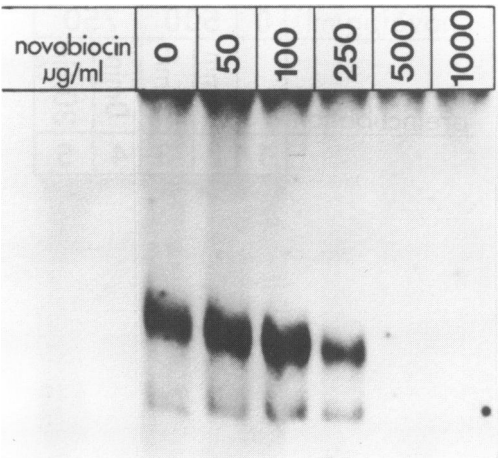

Figure 1. Effect of increasing novobiocin concentrations on 5S rRNA gene transcription. Transcription factors were added to the template DNA immediately after the addition of increasing amounts of novobiocin as indicated in the figure. RNA polymerase III and nucleoside triphosphates (NTPs) as well as α-^{32}P-GTP were added and the reaction incubated at 22°C for 40 minutes. The reactions were stopped, the RNA products extracted and resolved on a 12% polyacrylamide gel as described in Materials and Methods. The amount of specific transcript in each lane is as follows: 211, 235, 243, 121, 19, 37 cpm's, respectively. A photograph of the autoradiogram is shown in this and all the subsequent figures.

interfering with RNA polymerase III action directly (i.e. initiation and/or elongation) or with some earlier step in transcription, such as factor binding which we know can occur fairly rapidly in the yeast system.

In order to determine at which step in the transcription reaction novobiocin acted, we performed the following experiment. The template DNA and transcription factors were mixed and preincubated for zero or 30 minutes prior to the addition of novobiocin (0, 500, or 750 µg/ml) and RNA polymerase III. The results of this preincubation experiment are shown in Figure 2. In lanes 2 and 4, novobiocin (500 and 750 µg/ml, respectively) is present when the transcription factors are added to the template DNA. As in Figure 1, no transcription occurs in the presence of these concentrations of novobiocin. In contrast, when the trascription factors are allowed to associate with the template for 30 minutes in the absence of novobiocin (lanes 3 and 5), transcription can occur when novobiocin (500 or 750 µg/ml) and RNA polymerase III are added later. The preformed transcription complexes are resistant to the inhibitory effects of novobiocin (compare lanes 3 and 5 with lanes 2 and 4). Furthermore, since a normal amount of transcription is obtained with the

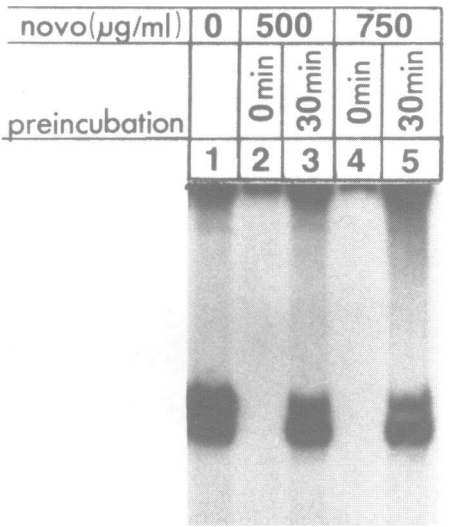

Figure 2. Preincubation of transcription factors with 5S rDNA decreases the inhibitory effect of novobiocin. Various amounts of novobiocin were added to the DNA prior to or 30 minutes after the addition of transcription factors. Lane 1, no novobiocin; lanes 2 and 3, 500 µg/ml at zero and 30 minutes, respectively; lanes 4 and 5, 750 µg/ml at zero and 30 minutes, respectively. Transcription was then assayed by the addition of RNA polymerase III and NTPs and incubation for 40 minutes at 22°C. Sample work-up was as indicated in Figure 1.

preincubated complexes (compare lane 1 with lanes 3 and 5), yeast RNA polymerase III must be fully capable of functioning in the presence of high concentrations of novobiocin. Thus, in agreement with the observations reported recently by Gottesfeld (6), novobiocin neither interferes with RNA polymerase III access to the complete factor-DNA complex nor does novobiocin appear to disrupt polymerase initiation or elongation.

Novobiocin Does Not Precipitate Transcription Factors

Cotten et al. (7) have shown that the novobiocin concentrations used to inhibit a putative eukaryotic DNA gyrase (and also to inhibit transcription in Xenopus oocytes and HeLa cell extracts) precipitate histones in vitro. Other proteins having a fairly high arginine content and/or overall positive charge can also be precipitated. All three yeast transcription factors appear to be quite basic. We expect that the yeast TFIIIA may have an arginine content similar to that of the Xenopus TFIIIA (3.5 mole %) and we have calculated the arginine content of yeast TFIIIB to be 4.4 mole % (Klekamp and Weil, manuscript submitted). No amino acid data are available at the present time for TFIIIC, but this protein does bind tightly to cation exchange resins. The

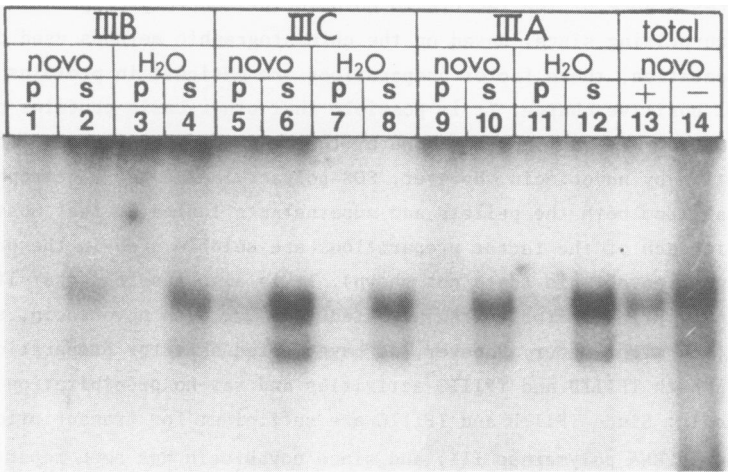

Figure 3. Novobiocin has no effect on the solubility of the individual transcription factors. Individual factors were incubated with 500 µg/ml novobiocin for 30 minutes at 0°C. The samples were then centrifuged (3 minutes 10,000 xg; Eppendorf centrifuge) to separate soluble (s) from precipitated (p) material. Precipitates were dissolved in transcription buffer containing the missing transcription factors, template DNA, RNA polymerase III and NTPs. Supernatants were added to a similar transcription mixure such that the final novobiocin concentration was 100 µg/ml. This amount of novobiocin has essentially no effect upon specific 5S rDNA transcription in vitro (see lanes 13 and 14 and also Figure 1). In lanes 1-4, precipitation of TFIIIB was assayed; in lanes 5-8, TFIIIC; in lanes 9-12, TFIIIA. Lane 13 represents the assay of the total transcription in the presence of 100 µg/ml novobiocin; lane 14, total transcription in the absence of novobiocin.

arginine contents and overall basicity of these protein factors make them potential candidates for precipitation by novobiocin. Since, in our studies, novobiocin seemed to act prior to RNA polymerase III association with the transcription complex, we thought perhaps novobiocin was removing one or more factors from the reaction by direct precipitation.

To test this idea, the individual factor preparations were incubated with novobiocin (500 µg/ml, or H_2O as a control) under normal transcription buffer conditions for 30 minutes at 0°C. The samples were then centrifuged at 10,000 xg and both the supernatants (s) and pelleted material (p) were assayed for factor activity after dilution to a final novobiocin concentration (100 µg/ml) which would permit normal transcription (see Figure 1). The results of this experiment are depicted in Figure 3. In comparing the transcriptional activity of the pellets (p) and supernatants (s) of each factor after exposure to novobiocin or H_2O (e.g. lanes 1 and 2 with 3 and 4, etc.), it is clear that under the conditions tested, all three transcription factors remained predominantly soluble.

The resistance of the factors to novobiocin-induced precipitation was somewhat surprising since, based on the chromatographic methods used during their preparation, these factor preparations are enriched in proteins having an overall positive charge. It is possible that other basic proteins present in these factor preparations may "spare" the transcription factors from precipitation by novobiocin. However, SDS-polyacrylamide gel electrophoresis of material from both the pellets and supernatants indicated that most of the proteins in each of the factor preparations are soluble even in the presence of 1000 µg/ml novobiocin (data not shown). It is also possible that if all three transcription factors were incubated together with novobiocin, some co-precipitation might occur. However, we have tested a factor preparation containing both TFIIIB and TFIIIC activities and saw no precipitation effect of novobiocin. Since TFIIIB and TFIIIC are sufficient for transcription of tRNA genes by RNA polymerase III, and since novobiocin has been reported to also inhibit tRNA gene transcription in vitro (6), we would presume that if novobiocin precipitation required the total transcription factor complex, TFIIIB and TFIIIC would have been sufficient for such an effect.

Novobiocin Inhibits TFIIIB Stable Association with 5SrDNA-TFIIIA-TFIIIC

In view of the observations above, it seemed likely that novobiocin was inhibiting 5S rDNA gene transcription by preventing or altering the appropriate factor-DNA interactions and/or factor-factor interactions required for stable transcription complex formation. In order to test these ideas further, we needed to be able to quickly isolate complexes that had been formed in the presence of novobiocin and ask if specific factors had failed to bind. We therefore made use of 5S rDNA bound to cellulose. This type of DNA preparation has been used previously to determine the kinetics of transcription complex formation (9). In our studies, the ability of various transcription factors to bind to 5SrDNA in the presence of novobiocin was directly examined in the following manner. After incubation, the samples of DNA-cellulose (with any factor(s) bound) were isolated by a quick (5 sec.) centrifugation, the novobiocin and unbound proteins were washed away, and the resulting complexes were assayed for transcriptional competence.

In our initial studies (without novobiocin, data not shown) we found that factors TFIIIA and TFIIIC associate with the 5S rRNA gene to form what is often refered to in other systems as a metastable complex. Factor TFIIIB required previous association of TFIIIA and TFIIIC with the DNA in order to become stably sequestered onto the template. This is consistent with results obtained by template competition experiments reported by Segall (13). It has been our experience that TFIIIB may be removed from the 5SrDNA-TFIIIA-TFIIIC-TFIIIB complex by washing the cellulose-bound material with buffer containing

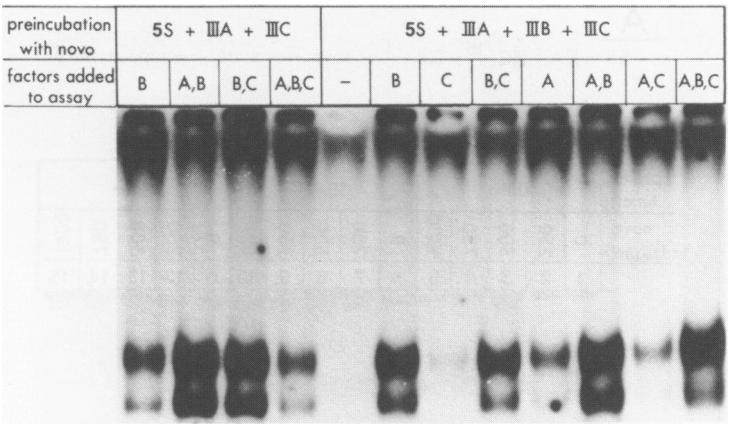

Figure 4. Partial but not complete factor-DNA complexes can form in the presence of novobiocin. The various transcription factors were incubated with cellulose-bound 5S rDNA in the presence of 500 µg/ml novobiocin. After washing with BC/100 to remove the novobiocin and unbound proteins, The resulting complexes were assayed for transcriptional activity by the addition of RNA polymerase III, NTPs and transcription factors as indicated.

10 mM Mg^{++} (data not shown). This observation may be similar to the instability of factor TFIIIB binding that was described by Carey et al (18). Nonetheless, a transcriptionally competent stable transcription complex consisting of factors TFIIIA, TFIIIB, and TFIIIC, can be isolated on the cellulose-bound 5S rDNA when a buffer not containing Mg^{++} is used in the washing procedure.

The effect of novobiocin on the formation of factor-DNA complexes is shown in Figure 4. In the first four lanes, TFIIIA and TFIIC were preincubated with the cellulose bound 5SrDNA in the presence of novobiocin. After isolation, the resulting complex is transcriptionally active upon addition of the complementing TFIIIB and RNA polymerase III. In the right hand eight lanes, the DNA-cellulose was preincubated with all three transcription factors in the presence of novobiocin. The resulting complex is transcriptionally inactive. If TFIIIB, alone or in combination with other factors, is added to the transcription assay (that includes RNA polymerase III) normal amounts of transcription are observed. TFIIIB activity can be recovered in the wash supernatant which presumably contains unbound material (data not shown), indicating that the factor had not been irreversibly inactivated by novobiocin. It is clear from an examination of the data shown that factors TFIIIA and TFIIIC are able to stably associate with the cellulose-bound 5S rDNA in the presence of 500 µg/ml novobiocin. The addition of TFIIIB to the

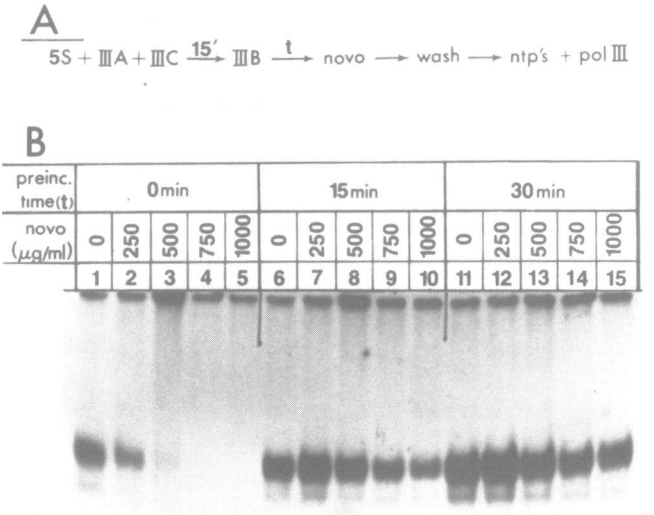

Figure 5. Association of TFIIIB with 5SrDNA-TFIIIA-TFIIIC results in formation of a novobiocin-resistant complex. An outline of the experiment is shown in panel A, transcription data in panel B. Cellulose-bound 5S rDNA was preincubated with TFIIIA and TFIIIC for 10 minutes at 22°C. TFIIIB was then added for 0 (lanes 1- 5), 15 (lanes 6-10), or 30 minutes (lanes 11-15) prior to the addition of novobiocin at the final concentrations indicated. The reactions were continued for a total incubation time of 90 minutes after which the complexes were washed and assayed for stable complex formation by the addition of RNA polymerase III and NTPs.

transcription assay complements the 5SrDNA-TFIIIA-TFIIIC complex and results in normal transcription of the gene. However, if factor TFIIIB (along with TFIIIA and TFIIIC) is added to the 5SrDNA-cellulose during the preincubation with novobiocin, TFIIIB cannot be stably sequestered onto the 5SrDNA-TFIIIA-TFIIIC complex. Factor TFIIIB must be added to the transcription assay in order for normal transcription to occur. Although the addition of TFIIIA and/or TFIIIC to the transcription assay allows for some transcription, the amount of transcription obtained is less than 20% of that obtained by the addition of TFIIIB alone (or in combination with TFIIA and/or TFIIIC). These data strongly suggest that TFIIIB cannot bind to a 5SrDNA-TFIIIA-TFIIIC in the presence of novobiocin. Since novobiocin does not appear to precipitate TFIIIB (Figure 3), we conclude that novobiocin somehow directly interferes with factor-factor interactions.

To test this hypothesis further, factors TFIIIA and TFIIIC were pre-incubated with the 5SrDNA-cellulose in the absence of novobiocin. Factor TFIIIB was then added for various lengths of time before different

concentrations of novobiocin were added (see Figure 5A for an outline of the experiment). All preincubation reactions were conducted for a total of 90 minutes before the complexes were washed and assayed for transcription by the addition of RNA polymerase III and nucleoside triphosphates (NTPs). The results of these transcription assays are shown in Figure 5B. In lanes 1 through 5, novobiocin was added to the 5SrDNA-TFIIIA-TFIIIC complex immediately before TFIIIB (zero minute preincubation with TFIIIB). In a manner similar to the results shown in Figure 1, novobiocin concentrations above 250 µg/ml inhibit 5S gene transcription. However, if TFIIIB is first allowed to associate with the 5SrDNA-TFIIIA-TFIIIC complex for 15 minutes (lanes 6-10) or 30 minutes (lanes 11-15), the resulting transcription complex is resistent to previously inhibitory concentrations of novobiocin as high as 1000 µg/ml. Assaying the transcriptional activity of the 5SrDNA-TFIIIA-TFIIIB-TFIIIC complex formed after 45 and 60 minute preincubations results in no further increase in transcription over that obtained after 15 or 30 minutes (data not shown), indicating that maximal amounts of stable transcription complexes had been formed. The parallel effects of novobiocin on overall transcription complex formation (Figures 1 and 2) and, more specifically, on factor TFIIIB association with preformed 5SrDNA-TFIIIA-TFIIIC complexes (Figure 5) lead us to conclude that novobiocin specifically inhibits TFIIIB binding to the 5SrDNA-TFIIIA-TFIIIC complex in an active form. The mechanism by which novobiocin acts to disrupt transcription complex formation is currently not known. Certainly though, the site or sites with which it interacts are hidden in the complete stable transcription factor complex, once it has formed.

DISCUSSION

Our studies have shown that novobiocin specifically interferes with transcription factor TFIIIB, disrupting stable transcription complex formation and thus eliminating any chance for transcription to occur. The data indicate that: 1) factors TFIIIA and TFIIIC are able to stably associate with 5S rDNA, forming a metastable complex with this gene, in the presence of novobiocin, 2) a preformed transcription complex (5S rDNA-TFIIIA-TFIIIB-TFIIIC) is resistant to the inhibitory effects of novobiocin, and 3) the kinetics of formation of this resistant complex are similar to that found for the stable association of factor TFIIIB to a preformed 5SrDNA-TFIIIA-TFIIIC complex. Amino acid analysis of purified TFIIIB indicates that the protein has an arginine content of approximately 4.4 mole %. Although this amount of arginine, in combination with its overall basicity, makes it likely that it might be directly precipitated by novobiocin, our data indicate that protein precipitation is

probably not the mechanism by which novobiocin inhibits 5S rRNA gene transcription in yeast.

We cannot rule out the possibility that novobiocin may still associate with TFIIIB (or the other factors) in some disruptive manner. It is possible that novobiocin may bind to the factor(s) and sterically hinder important interactions between factor TFIIIB and the 5SrDNA-TFIIIA-TFIIIC complex. These interactions could be through specific sites on factor TFIIIB itself and/or through sites exposed along the TFIIIA-TFIIIC surface. Novobiocin could also be acting to prevent some conformational change that factor TFIIIB and/or the TFIIIA-TFIIIB-TFIIIC complex must undergo to lock TFIIIB into place and form a stable complex that can be recognized by RNA polymerase III. These ideas are quite possible since novobiocin is able to disrupt histone octamer formation (i.e. protein-protein interactions) at novobiocin concentrations of 25-50 µg/ml, well below those concentrations that precipitate the individual histones (14).

Novobiocin was initially characterized as a competitive inhibitor of ATP binding to bacterial gyrase. This binding inhibits the subsequent action of bacterial gyrase (see Refs. 4, 5 and references therein). There has been only one report that reliably demonstrates the presence of a gyrase activity in eukaryotic cells. Thompson and Mosig (19) have reported a DNA gyrase activity in Chlamydomonas that seems to be associated with expression of specific chloroplast genes. This activity is readily inhibited by concentrations of novobiocin less than 0.1 µM (< 0.1 µg/ml). Inhibition by these novobiocin concentrations is consistent with the effect of novobiocin on the bacterial gyrase. However, the concentrations of novobiocin used to inhibit the putative gyrase in other eukaryotes is many orders of magnitude above this (1,2,3). Although it is quite possible that a DNA gyrase exists in these and other eukaryotic systems, we cannot detect any gyrase activity in our in vitro transcription system. Yet, high concentrations of novobiocin clearly inhibit in vitro transcription of the yeast 5S rRNA gene. Our data strongly suggest that this inhibition is a consequence of the prevention of TFIIIB from forming a stable complex with a metastable 5SrDNA-TFIIIA-TFIIIC complex.

Hazuda and Wu (20) have recently reported a DNA-activated ATPase activity associated with the Xenopus TFIIIA. The activity of this ATPase is quite low (V_{max} of 1.7 nmol/min/mg protein) and is similar to that reported for SV40 large T antigen (21). If this ATPase activity were required for 5S rRNA gene transcription, and if novobiocin inhibited the ATPase, we might have an explanation for the high concentrations of novobiocin required for inhibition of class III transcription. However, based on our data, we would have to conclude that the ATPase activity were required not for TFIIIA function alone,

but either for TFIIIB binding or for some conformational change, etc. that requires the presence of all three transcription factors. Moreover, inhibition of an ATPase associated with TFIIIA by novobiocin would not explain the inhibition of tRNA transcription by novobiocin (6). Novobiocin would have to either sterically inhibit factor-factor interactions, perhaps involving arginine residues, or there would have to be some other nucleotide binding site associated with TFIIIB and/or TFIIIC to explain the inhibition of all class III gene transcription by novobiocin.

The effect of novobiocin on transcription complex formation might be due to its ability to associate with some site(s) in common with other nucleotides which have been postulated to bind within the transcription complex (22). If this were the case, these nucleotides (ATP, GTP, dATP,etc.) might allow for stable complex formation (and, in some systems, even stimulate it), while novobiocin would inhibit it. The structures of these compounds are sufficiently different to postulate this differential behavior. However, there does not appear to be an absolute requirement for any nucleotide binding during stable transcription complex formation in yeast since such a complex can be formed in the absence of nucleotides (as in the experiments presented here). In fact, our own studies (10) and the data reported recently by Segall (13) indicate that there is little or no lag in yeast 5S rRNA gene transcription in vitro. These results make it difficult to predict and/or invoke any role for nucleotides in the formation of yeast class III gene stable transcription factor complexes, and henceforth develop a more detailed model for the inhibitory action of novobiocin in this reaction. It appears clear, however, that high concentrations of novobiocin can interfere with protein complex formation without precipitating the proteins involved.

Although novobiocin is definitely an inhibitor of bacterial gyrase, its use in the study of eukaryotic transcription reactions must be approached with caution. Use of novobiocin at such high concentrations may be helpful, but it also makes for experiments that are potentially subject to artifacts. Our understanding of how the various class III gene transcription factors bind and interact with specific genes and with one another is at the present time vague enough such that we should avoid introducing more complications into the data interpretation than already exist.

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