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Novobiocin inhibits initiation of RNA polymerase II-directed transcription of the mouse metallothionein-I gene independent of its effect on DNA topoisomerase II

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ABSTRACT

The requirement for ATP hydrolysis in the initiation of RNA polymerase II (Pol II)-directed transcription and the relationship between ATP and novobiocin action led us to investigate whether novobiocin could inhibit transcription of the mouse metallothionein-I (MT-I) gene. Novobiocin inhibited the MT-I gene transcription in a fractionated rat hepatoma nuclear extract in a dose-dependent manner by direct interaction with a nuclear factor(s). This interaction prevented formation of stable preinitiation complexes but did not affect elongation of MT-I mRNA. Preincubation of the nuclear extract with ATP prevented the action of novobiocin on MT-I gene transcription. Although novobiocin is known to inhibit DNA topoisomerase II, VM-26, a specific inhibitor of this enzyme had no effect on the transcription. These results indicate that novobiocin blocks the Pol II-directed transcription by inhibiting formation of preinitiation complexes at an ATP-dependent step.

INTRODUCTION

Eukaryotic RNA polymerases do not accurately initiate gene transcription in the absence of specific cellular transcription factors (see ref. 1). In the case of RNA polymerase II (Pol II)-directed transcription, chromatographic fractionation of HeLa cell extracts has led to the resolution of at least 5 fractions (TFIIA-TFIIE) which are necessary for specific transcription of the adenovirus 2 major late promoter (2,3). In the presence of RNA polymerase II and a class II promoter containing gene, these factors associate to form a stable preinitiation complex (4). Although considerable progress has been made on the purification of specific factors involved in Pol II-mediated transcription and the order of interaction of the various protein and DNA components (5,6), the precise role of these factors in initiation of transcription has not been completely elucidated. Specific inhibitors of the events leading to the formation of stable preinitiation complexes would be valuable tools in the elucidation of the mechanism of this critical cellular process.

Recently, dichlororibofuranosylbenzimidazole (DRB) has been shown to

interfere with Pol II-directed transcription by inhibiting the activity of a casein kinase II (7,8). The dependence of the class II gene transcription on the protein kinase is consistent with earlier observations that ATP hydrolysis is a prerequisite for accurate initiation of Pol II-directed transcription (9,10). Recent studies have shown that an ATP dependent step in preinitiation complex formation in the RNA polymerase III-directed transcription of cloned 5S and tRNA genes can be blocked by the drug novobiocin (11). Since the action of novobiocin is related to ATPase activity (12) and ATP hydrolysis is required for specific initiation of transcription by RNA polymerase II (9,10), it was of considerable interest to determine whether novobiocin could inhibit initiation of transcription of a specific class II gene. To test this possibility, we studied transcription of a cloned mouse metallothionein-I gene in a fractionated nuclear extract. In this report we demonstrate that novobiocin inhibits metallothionein gene transcription at the initiation step. Moreover, this inhibition is not mediated by DNA topoisomerase II which is known to be sensitive to this drug.

METHODS AND MATERIALS

Plasmid

The mouse metallothionein-I (MT-I) minigene cloned into pUC18 (a generous gift from Dr. Richard Palmiter) was used in these experiments. This minigene has several unique restriction sites (Figure 1). We have previously shown that *in vitro* transcription from the MT-I minigene linearized with Bgl II results in an accurately initiated, 67 nucleotide-long transcript (13). *In vitro* transcription of the MT-I minigene linearized with Sst II produces the expected 234 nucleotide-long product.

Enzyme Extract

Nuclear extract from solid hepatoma tissue containing RNA polymerase II and Pol II transcription factors was prepared as previously described (13,14). Briefly, nuclear extract from Morris hepatoma 3924A was fractionated on a DEAE-Sephadex column. The fraction containing RNA polymerase II and essential Pol II transcription factors (DE-C) eluted with 300 mM $(NH_4)_2SO_4$. Fraction DE-C was precipitated with 0.42 g $(NH_4)_2SO_4$/ml, dialyzed and stored at -70°C until use. Enzyme extract prepared in this manner was generally stable for at least 6 months as long as the aliquoted enzyme was not repeatedly thawed and refrozen.

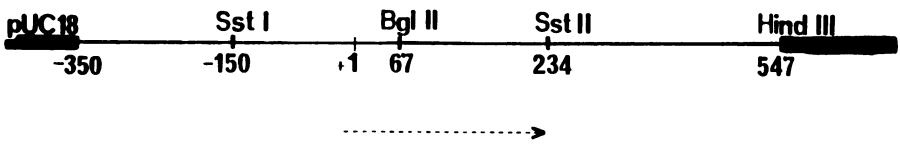

Figure 1:
Restriction map of the mouse metallothionein-I minigene cloned into pUC18. The heavy black lines denote vector sequences, the thin black line denotes gene sequences and the dashed lines denotes mRNA transcript.

Transcription Assay

In vitro transcription was performed according to standard methodology. Reaction mixtures contained 15 mM Hepes (pH 7.9), 10% glycerol, 1 mM DTT, 48 mM KCl, 0.1 mM EDTA, 4 mM $MgCl_2$ and 50-500 μM unlabeled nucleotides. Total reaction volume was 25 μl. RNA synthesized in vitro was extracted and electrophoresed on 8% polyacrylamide/8M urea gels. Transcripts were visualized by autoradiography and quantitated by densitometry (Zeineh Soft Laser).

RNA Polymerase and Protein Kinase Assay

RNA polymerase and protein kinase activities were determined as described (15,16).

Chemicals and Inhibitors

Nucleotides were from Calbiochem, restriction enzymes from BRL and novobiocin from Sigma. Camptothecin and VM-26 were generous gifts from Drs. Leroy Liu and Joel Gottesfeld, respectively.

RESULTS

Effect of Novobiocin on MT-I Gene Transcription

The effect of novobiocin on MT-I gene transcription was examined by varying the concentration of the antibiotic in the transcription reaction. Accurately initiated transcription from the Bgl II-cleaved MT-I gene yields a product 67-nucleotides long (13). During the early phase of experimentation, we found that fraction DE-C also supported faithful transcription of MT-I DNA cleaved with Sst II. Transcription of MT-I DNA resulted in a 234 nucleotide-long product which is the expected size of the run-off transcript (17). A second major band of approximately 400 nucleotides was also observed in this transcription system. This product was derived from the pUC18 vector since it was absent when transcription

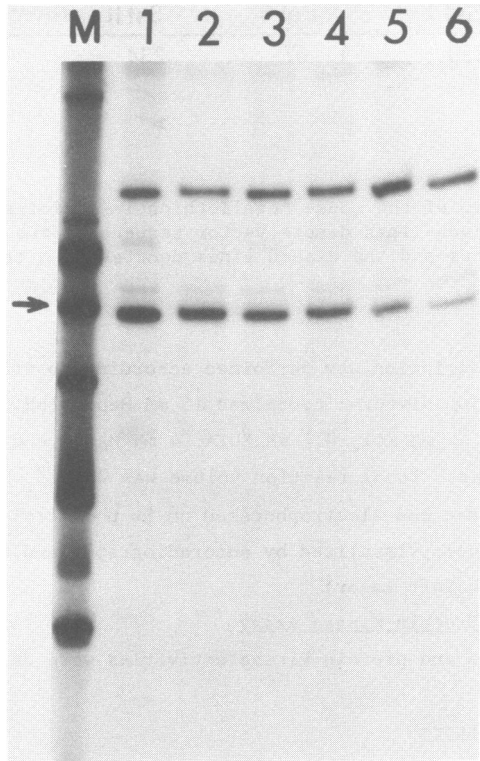

Figure 2:
Effect of novobiocin on in vitro transcription from the Sst II-cleaved metallothionein-I gene. Lanes 1-6 included 0, 0.1, 0.25, 0.5, 1 and 2 mM novobiocin, respectively. Transcription reactions contained 300 ng of DNA and 16 μg of fraction DE-C. DNA markers (M) are the 5' end-labeled Hae III digest of ϕX174. The arrow corresponds to the 234-nucleotide-long ϕX174 DNA fragment.

was performed using MT-I gene fragment in the absence of vector (data not shown). Novobiocin inhibited transcription of the 234 nucleotide-long product from the Sst II-linearized MT-I gene template (Figure 2) in a dose-dependent manner. Quantitation by densitometric scanning of the autoradiogram revealed that approximately 50% inhibition occurred at 250 μM novobiocin and 85% inhibition at 2 mM novobiocin. Following electrophoresis, the 234 nucleotide transcript was excised from the gel matrix, dissolved in scintillation cocktail and counted. Under standard assay conditions, approximately 0.013 moles of transcript/mole of template were synthesized. This is consistent with other in vitro RNA polymerase II

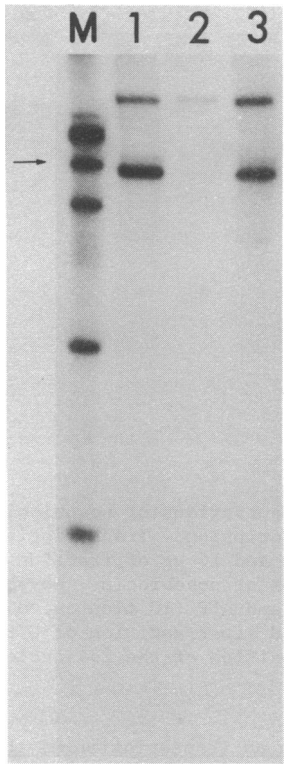

Figure 3:
Site of novobiocin action on in vitro metallothionein-I gene transcription. Transcription reactions contained 300 ng of Sst II cleaved metallothionein-I DNA and 16 μg of fraction DE-C. Lane 1: no novobiocin. Lane 2: novobiocin (1 mM) was preincubated (10 minutes, 30°C) with DNA, DE-C and the nucleoside triphosphates GTP, UTP and ATP. CTP was added at the end of this preincubation period and transcription continued for 20 minutes at 30°C. Lane 3: novobiocin (1 mM) and CTP were added after the preincubation reaction. Transcription continued for 20 minutes at 30°C. Markers (M) are as in Figure 2. The arrow denotes the position of the 234-nucleotide-long DNA fragment (see Fig. 2).

transcription systems (18). Quantitation of transcript produced per template generally corresponded to densitometric quantitation. In the presence of 2 mM novobiocin, substantially less transcript (0.002 moles) was produced. MT-I transcription inhibition was not due to precipitation of an essential transcription component, as the supernatant obtained after preincubation in the presence of the drug followed by high speed centrifugation retained the ability to transcribe MT-I DNA (data not shown).

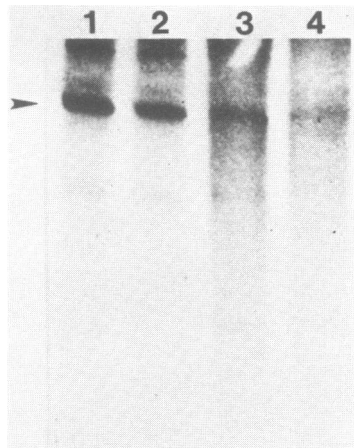

Figure 4:
Effect of varying concentrations of novobiocin on the initiation of metallothionein-I gene transcription. Transcription reactions contained 300 ng of Sst II-cleaved DNA and 16 μg of fraction DE-C. Lanes 1-4 included 0, 0.25, 0.5 or 1 mM of novobiocin. Novobiocin was preincubated with DNA, DE-C and GTP, UTP and ATP (10 minutes, 30°C) before addition of CTP. Transcription continued after addition of CTP for 20 minutes at 30°C. The arrowhead denotes the position of the 234-nucleotide long DNA fragment (see Fig. 2).

Site of Action of Novobiocin on Transcription of the MT-I Gene

The site at which novobiocin acts in the Pol II-directed transcription of the mouse MT-I gene was determined by adding novobiocin at different stages of transcription, specifically during either the initiation or chain elongation reactions. This experiment was performed by including novobiocin during or after formation of the first phosphodiester bond of the MT-I mRNA chain. Since the first four nucleoside triphosphates incorporated into the MT-I mRNA are GTP, UTP, CTP and ATP (17), CTP was withheld. ATP was included in the reaction in order to provide for a potential ATP requirement during initiation complex formation. This reaction produced a preinitiation complex comprised of RNA polymerase II, pol II factors, metallothionein promoter sequences and the first phosphodiester bond (G--U). Initiation of transcription can occur but elongation of the mRNA chain cannot proceed in the absence of the remaining nucleoside triphosphate (CTP). Addition of CTP after the preincubation period resulted in the production of the 234 nucleotide-long transcript (Figure 3, lane 1). The presence of novobiocin during the formation of the

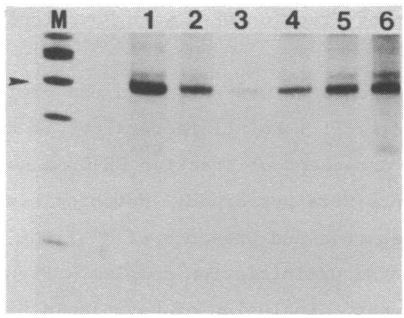

Figure 5:
Effect of preincubation of novobiocin with fraction DE-C in the absence of the nucleotides on metallothionein-I gene transcription. Lane 1: Transcription in the absence of novobiocin; Lane 2: Transcription in the presence of novobiocin (1 mM). The reactions were carried out at 30°C. Lanes 3-6 represent the addition of novobiocin before, during or after stable preinitiation complex formation. The first arrow represents 10' preincubation at 30°C and the second arrow denotes 30' incubation at 30°C (NTPs: nucleoside-triphosphates):

Lane 1 DE-C + DNA + NTPS --> -->
Lane 2 DE-C + DNA + NTPS + novobiocin --> -->
Lane 3 DE-C + novobiocin --> MT-I DNA + NTPs -->
Lane 4 MT-I DNA + novobiocin --> DE-C + NTPs -->
Lane 5 MT-I DNA + DE-C + novobiocin --> NTPs -->
Lane 6 MT-I DNA + DE-C --> novobiocin + NTPs -->

first phosphodiester bond inhibited MT-I gene transcription, whereas the presence of the drug during the elongation reaction had no effect on transcription (Figure 3, lane 2 vs. lane 3). This experiment clearly demonstrates that novobiocin acts at the level of initiation of mouse MT-I gene transcription.

The effect of novobiocin on transcription initiation was further examined by performing a dose-response experiment specifically during the initiation reaction. It can be seen that increasing the concentration of novobiocin present during the initiation reaction inhibits production of the 234 nucleotide long transcript (Figure 4). Densitometric analysis revealed that approximately 50% inhibition was achieved at 250 μM novobiocin. This result is similar to that obtained when novobiocin was added during the complete reaction (see Figure 2) confirming that nearly 100% of the novobiocin effect occurred during the initiation of transcription.

Effect of Preincubation of Fraction DE-C with Novobiocin on MT-I Gene Transcription

To determine if novobiocin was acting on the preinitiation complex (MT-I DNA + RNA polymerase II + Pol II factors) or if the antibiotic was acting directly with a component of fraction DE-C, a series of preincubation experiments were performed. Novobiocin was preincubated with fraction DE-C in the absence and presence of MT-I DNA, i.e. before, during and after formation of the preinitiation complex. Figure 5 shows the results of this experiment. Inclusion of novobiocin prior to or during formation of stable preinitiation complexes (lanes 2-5) inhibited transcription. The greatest extent of inhibition was obtained when novobiocin was preincubated with fraction DE-C (compare lanes 2-5). However, when novobiocin was added after formation of preinitiation complexes, much less inhibition of transcription was observed (lane 6). These results suggest that novobiocin inhibits transcription initiation by blocking formation of stable preinitiation complexes possibly by direct interaction with a component of fraction DE-C.

Effect of ATP on Novobiocin Inhibition of MT-I Gene Transcription

It is known that the initiation of RNA polymerase II-mediated

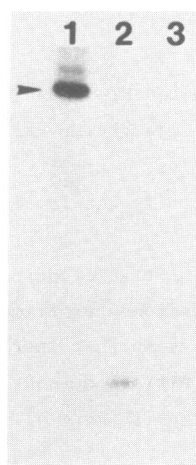

Figure 6:
Role of ATP hydrolysis in the *in vitro* transcription of metallothionein-I. Transcription reactions contained 300 ng of Sst II-cleaved MT-I DNA and 16 μg of fraction DE-C. Transcription reactions contained 500 μM ATP (lane 1), 500 μM AMP-PNP (lane 2), neither ATP nor AMP-PNP (lane 3).

transcription is dependent upon ATP hydrolysis (9,10) which is probably occurring at a later stage of the initiation reaction (2). We, therefore, investigated whether the transcription of MT-I is dependent on ATP hydrolysis. The ATP analog AMP-PNP, which has a non-hydrolyzable β-γ phosphate bond, was substituted for ATP at equimolar concentrations. As seen in Figure 6, transcription in the presence of ATP resulted in the

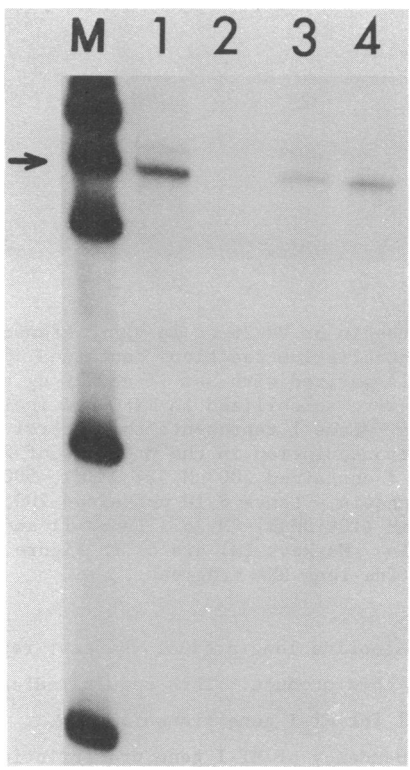

Figure 7:
Effect of ATP on the novobiocin inhibition of the in vitro transcription of metallothionein-I. Transcription reactions contained 300 ng of Sst II-cleaved metallothionein-I DNA and 16 μg of fraction DE-C. Lane 1 represents the control transcription. Lane 2 is the resultant transcription when novobiocin is preincubated (10 minutes, 30°C) with fraction DE-C in the absence of ATP. Lanes 3 and 4 are the transcription when 50 or 500 μM, respectively, of ATP is preincubated (10 minutes, 30°C) with fraction DE-C in the absence of novobiocin. Following preincubation, the remaining appropriate components of the transcription reactions were added and transcription proceeded for 30 minutes at 30°C. Markers (M) are as in Figure 2. The arrow denotes the position of the 234-nucleotide-long DNA fragment.

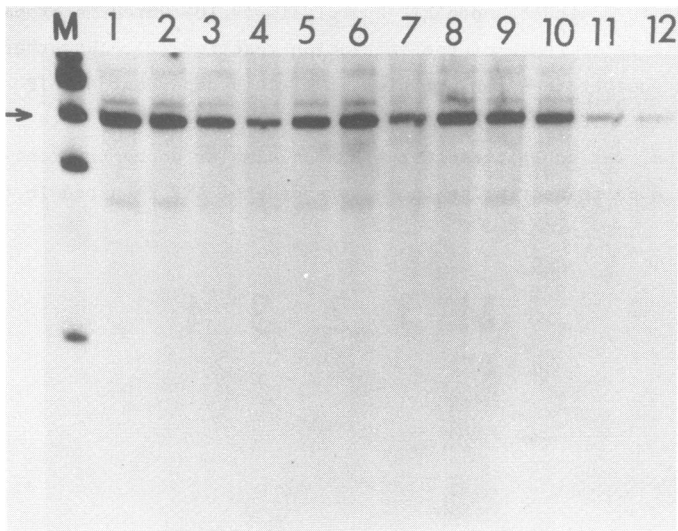

Figure 8:
Effect of camptothecin or VM-26 on in vitro transcription of metallothionein-I. Transcription reactions contained 300 ng of metallothionein-I DNA linearized with Sst II and 16 μg of fraction DE-C. Camptothecin and VM-26 were solubilized in DMSO and included in the transcription reactions. Lane 1 represents the control transcription and lanes 2-4 the control transcription in the presence of 2, 5 or 10% DMSO, respectively. Lanes 5-7 contained 200 μM (2% DMSO), 500 μM (5% DMSO) or 1 mM (10% DMSO) camptothecin. Lanes 8-10 contained 200 μM (2% DMSO), 500 μM (5% DMSO) or 1 mM (10% DMSO) VM-26. Lanes 11 and 12 contained 0.5 and 1.0 mM of novobiocin. Markers (M) are as in Figure 2. The arrow denotes the 234-nucleotide-long DNA fragment.

appearance of a 234-nucleotide long product whereas, replacement of ATP with AMP-PNP abolished this product. This result indicates that ATP hydrolysis is essential for MT-I gene transcription.

Given the ATP-dependency of MT-I gene transcription and the fact that novobiocin is a competitive inhibitor of ATP with regard to its activity on DNA topoisomerase II (12), we next sought to determine if novobiocin was competing with ATP in the RNA polymerase II-directed transcription process. To examine this possibility, the enzyme extract was preincubated (10 minutes, 30°C) with ATP before or after exposure to novobiocin. The results of this experiment are shown in Figure 7. Preincubation of fraction DE-C with novobiocin prior to the addition of ATP resulted in dramatic inhibition of RNA polymerase II-directed transcription of MT-I. In contrast, preincubation of fraction DE-C with ATP prior to addition of

novobiocin significantly reduced the extent of inhibition. When the level of ATP was increased from 50 μM to 500 μM, the novobiocin inhibition was further reduced but not eliminated. Concentrations of ATP as high as 1 mM did not enhance this effect (data not shown). These results indicate that novobiocin inhibits Pol II-directed transcription by inhibiting an ATP-dependent reaction.

Is the Inhibition of Transcription by Novobiocin Mediated by DNA Topoisomerase II?

Since novobiocin is an inhibitor of eukaryotic DNA toposiomerase II (12), we then investigated if the mechanism of novobiocin inhibition of transcription was mediated by this enzyme. The use of linear templates in our experiments made it unlikely that the novobiocin effect on transcription was mediated via a topoisomerase-induced relaxation of the DNA template. However, to rule out topoisomerase involvement conclusively, MT-I gene transcription was performed in the presence of specific inhibitors of DNA topoisomerases I and II. Specific inhibition of topoisomerase I and II is achieved with 100-200 μM camptothecin and VM-26, respectively (19,20). If topoisomerase I or II were involved in MT-I gene transcription, inclusion of camptothecin or VM-26 would inhibit the transcription. Since camptothecin and VM-26 were dissolved in DMSO, the corresponding controls for DMSO in the transcription reaction were included (lanes 2-4). Concentrations of camptothecin and VM-26 up to 1 mM had no effect on the transcription (compared to the appropriate DMSO control) while 1 mM novobiocin clearly inhibited transcription of the mouse MT-I gene (Figure 8).

DISCUSSION

The present study indicates that novobiocin inhibits RNA polymerase II-mediated MT-I gene transcription specifically at the initiation phase of the transcription reaction; addition of novobiocin at the RNA elongation step had no effect on MT-I gene transcription. This is consistent with the site of action of novobiocin on RNA polymerase III-directed transcription of 5S (21) and tRNA (11) genes. Novobiocin also inhibited RNA polymerase I-directed transcription of rDNA by a similar mechanism (M.L. Webb and S.T. Jacob, unpublished data).

Dichlororibofuranosylbenzimidazole (DRB) is another compound which inhibits RNA polymerase II-directed *in vitro* transcription at the initiation step (7,8,22). DRB has been shown to inhibit the transcription

of the adenovirus and human β-globin genes (7). Preliminary data also indicate a similar effect on the mouse MT-I gene transcription (M.L. Webb and S.T. Jacob, unpublished data). However, the mode of action of novobiocin is distinct from that of DRB. DRB acts via specific inhibition of a cyclic nucleotide independent kinase (8), whereas novobiocin has no effect on the nuclear form of cyclic nucleotide independent kinase (NII) (M.L. Webb and S.T. Jacob, unpublished data). Moreover, unlike novobiocin, DRB does not inhibit RNA polymerase III- (7) or RNA polymerase I- (M.L. Webb and S.T. Jacob, unpublished data) directed in vitro transcription. Therefore, DRB is specific for RNA polymerase II-specific transcription whereas novobiocin acts via a more general transcription mechanism. These studies suggest that there are two ATP-dependent reactions in the initiation of RNA polymerase II-directed transcription; one sensitive to DRB and the other to novobiocin.

The novobiocin effect on MT-I gene transcription is not mediated by DNA topoisomerase I or II as the specific topoisomerase inhibitors camptothecin and VM-26 did not block transcription of the MT-I gene. This finding is similar to that observed in RNA polymerase III-directed transcription where camptothecin and VM-26 are not required for tRNA gene transcription (11). Similarly, Broyles and Moss (23) also demonstrated that DNA topoisomerase was not involved in the novobiocin inhibition of vaccinia virus RNA polymerase-directed transcription of the vaccinia growth factor gene. Thus, it is apparent that novobiocin-induced transcriptional inhibition is mediated through another target(s).

One potential candidate for the novobiocin target is an ATPase as the action of novobiocin is related to ATPase activity (12) and ATP hydrolysis is essential for initiation of Pol II-directed transcription (9,10). Recently, Reinberg and Roeder (2) reported the purification of a trans-factor, TFIIE, which exhibits DNA-dependent ATPase activity. Novobiocin has been shown to inhibit a DNA dependent ATPase activity in vaccinia virus extracts (23). These observations are consistent with the proposal that the novobiocin effect on Pol II-directed transcription may be mediated through an ATPase activity. However, this proposed mechanism fails to explain all of the novobiocin effects on RNA polymerase III-directed transcription. An ATPase activity is associated with Xenopus factor TFIIIA (24) and novobiocin can prevent association of this factor with 5S and VA RNA gene promoters. On the other hand, TFIIIB and TFIIIC do not appear to exhibit ATPase activity and yet novobiocin prevents their association with

protein (21) and DNA (25) respectively. The multiplicity of transcription systems and trans-factors which are affected by novobiocin suggests that novobiocin is a general inhibitor of transcription by preventing DNA-protein or protein-protein interactions necessary for formation of stable initiation complexes.

Finally, the concentrations of novobiocin used in this study deserve comment. Although these concentrations are in the range of 0.25 mM to 1 mM, they are consistent with those used previously to inhibit transcription (11,21,23). In addition, we observed non-linear dose-dependent relationships between novobiocin and the transcription inhibition of MT-I DNA truncated with Sst II. Thus, it is possible that novobiocin is a less potent form of a class of drugs which act preferentially on essential factors involved in transcription initiation. Nonetheless, novobiocin and other drugs which act at different stages of transcription, could prove valuable tools for dissecting and ultimately understanding the sequence of events leading to the production of a functional transcript.

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