The effect of novobiocin, a coumarin class antibiotic, on rat ribosomal gene (rDNA) transcription in a fractionated extract derived from adenocarcinoma ascites cells (fraction DE-B) was studied. This drug inhibited transcription of rDNA by blocking initiation of transcription, whereas it had no effect on the elongation of the rRNA transcript. Order of addition experiments indicated that the novobiocin effect was at a step(s) in preinitiation complex formation. Preincubation of fraction DE-B with ATP before exposure to this antibiotic prevented inhibition of rDNA transcription. Since novobiocin has been shown to inhibit RNA polymerases II- and III-directed transcription of linear DNAs by interfering with a step(s) in the initiation reaction, these data suggest that initiation of transcription of all classes of RNA is inhibited by novobiocin by a mechanism independent of its effect on DNA topoisomerase II.

We have been studying the events leading to accurate initiation of gene transcription in eukaryotes. One approach to this problem is to identify specific inhibitors that can act at a defined stage of the initiation reaction. Previous studies have shown that novobiocin, a DNA gyrase and topoisomerase II inhibitor, blocks TFIIA'-dependent gyration and transcription of 5 S RNA gene chromatin (1, 2). More recently, we and others have examined the effects of novobiocin on RNA polymerase II (Pol II)-directed transcription of mouse metallothionein-I (MT-I) (3) and RNA polymerase III (Pol III)-directed transcription of the rRNA (4) and 5 S RNA genes (5, 6). Novobiocin inhibited initiation of Pol II and Pol III transcription by mechanisms independent of the unwinding activity of DNA topoisomerase II (3, 4). Subsequent investigations with purified Pol III trans-factors and 5 S RNA gene have indicated that novobiocin interrupts DNA-protein (6, 7) or protein-protein (5) interactions essential for transcription initiation.

In the present study, we have examined the effects of novobiocin on the RNA polymerase I-directed transcription of ribosomal RNA gene (rDNA). These data have shown that, similar to the Pol II and Pol III transcription systems, novobiocin blocked rDNA transcription at the initiation step and that this inhibition was independent of the relaxation activity of DNA topoisomerase II.

EXPERIMENTAL PROCEDURES

Plasmid—The plasmid pDJ4-3'ΔSK containing the rat ribosomal RNA gene inserted into pBR322 was described in detail elsewhere (8). This plasmid contains a 174-bp upstream element of the rat rDNA spacer located between -2357 and -2183 bp ligated to the -2183-bp position of rDNA. The 174-bp upstream element has been shown to exhibit characteristics of a Pol II enhancer (8). Transcription of Xhol-linearized pDJ4-3'ΔSK results in a 635-nucleotide-long RNA transcript (see Ref. 8).

Preparation of Extracts—Whole cell extract was prepared from adenocarcinoma ascites cells as described (9). These lysates were fractionated by DEAE-Sephadex chromatography to obtain the fraction (DE-B) which accurately initiated transcription of the rat rRNA gene (9). Protein concentration was determined by the method of Shaffner and Weissman (10).

In Vitro Transcription Assay—In vitro transcription was performed essentially as described (9). RNA synthesized in vitro was extracted and electrophoresed on 4% polyacrylamide/7 M urea gels. The products were visualized by autoradiography and quantitated by densitometric scanning (Zeineh Soft Laser).

RESULTS

Effect of Novobiocin on rDNA Transcription—The effect of novobiocin on rDNA transcription was studied by varying the concentration of the drug in the transcription reaction. Novobiocin inhibited transcription of Xhol-linearized pDJ4-3'ΔSK in fractionated (fraction DE-B) whole cell extract in a dose-dependent manner (Fig. 1). Quantitation by densitometric scanning of the autoradiograms revealed that 0.25 and 0.5 mM novobiocin inhibited transcription approximately 35 and 70% in fraction DE-B, respectively. Increasing the concentration of the drug to 2 mM completely abolished transcription. The possibility that novobiocin inhibited rDNA transcription by precipitating essential transcriptional components was examined by a preincubation experiment similar to that described for Pol III-directed transcription (5, 7). The results of this experiment (data not shown) demonstrated that the essential rDNA transcription components remained soluble after treatment with novobiocin.

The effect of novobiocin on rDNA transcription in fraction DE-B was further analyzed by excising the transcript from the gel matrix following electrophoresis. The radioactivity in the band was determined and the transcription was quantitated by densitometric scanning (see "Experimental Procedures" and Table I).
Inhibition of rDNA Transcription by Novobiocin

**FIG. 1.** Effect of novobiocin on rDNA transcription. Transcription reactions contained 240 ng of XhoI-cleaved rDNA and 15 μg of fractionated whole cell extract (fraction DE-B). Novobiocin was included in the reactions in lanes 1–5 at 0, 0.25, 0.5, 1, and 2 mM concentration, respectively. The arrowhead corresponds to the 635-nucleotide-long rDNA transcript.

**TABLE I**

Effect of novobiocin on the amount of rRNA produced per mole of rDNA

<table>
<thead>
<tr>
<th>Novobiocin (mM)</th>
<th>Transcript (mol/mol template)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.024</td>
</tr>
<tr>
<td>0.25</td>
<td>0.016</td>
</tr>
<tr>
<td>0.5</td>
<td>0.008</td>
</tr>
<tr>
<td>1.0</td>
<td>0.005</td>
</tr>
<tr>
<td>2.0</td>
<td>0.001</td>
</tr>
</tbody>
</table>

**Site of Action of Novobiocin on rDNA Transcription**—The site at which novobiocin acts in polymerase I-directed transcription was determined by adding novobiocin during the initiation (those steps up to and including the formation of the first phosphodiester bond) or elongation (those steps subsequent to formation of the first phosphodiester bond) phases of the transcription reaction. This experiment was performed by adding novobiocin during or after formation of the first phosphodiester bond of the rRNA transcript. Since the first three ribonucleoside triphosphates in rRNA are GCU (12), UTP was withheld. In this way an initiation complex was formed between RNA polymerase I, Pol I factors, rDNA promoter sequences, and the first two ribonucleoside triphosphates (GC). Elongation of the rRNA transcript could not occur until the remaining ribonucleoside triphosphate, UTP, was added. As shown in Fig. 2, the presence of novobiocin during the initiation reaction (lane 2) inhibited rDNA transcription, whereas the presence of the antibiotic during elongation (lane 3) had a minimal effect on transcription.

To elucidate further the novobiocin site of action, the drug was added before, during, or after formation of the rDNA preinitiation complex. Novobiocin was preincubated with fraction DE-B in the absence and presence of rDNA. Inclusion of novobiocin prior to or during formation of stable preinitiation complexes (Fig. 3, lanes 1–3) inhibited transcription, whereas addition of novobiocin after formation of preinitiation complexes (Fig. 3, lane 4) had no effect on rDNA transcription. These results demonstrate that novobiocin inhibits transcription initiation by blocking formation of stable preinitiation complexes.

**Effect of ATP on Novobiocin-induced Inhibition of rDNA Transcription**—Since novobiocin and ATP are related with respect to binding to DNA topoisomerase II (13), we next examined the relationship between ATP and novobiocin in the transcription of rDNA. In this experiment, fraction DE-B was preincubated (10 min at 30°C) with ATP or novobiocin prior to addition of the remaining components of the transcription reaction. Preincubation of fraction DE-B with the drug resulted in complete inhibition of transcription (Fig. 4, lane 2). In contrast, preincubation of fraction DE-B with ATP prevented the novobiocin-induced inhibition of rDNA transcription (Fig. 4, lanes 3 and 4). This effect was specific for ATP as preincubation of fraction DE-B with CTP, GTP, or UTP did not prevent the novobiocin inhibition of transcription (data not shown).

**DISCUSSION**

It is clear from this investigation that novobiocin prevents formation of stable preinitiation complexes by acting at an ATP-dependent step in the formation of such complexes. This is similar to the mechanism elucidated for the novobiocin-induced inhibition of transcription of metallothionein-I (3) and tRNA genes (4). In the RNA polymerase III system, where novobiocin actions have been studied in more detail due to the availability of purified trans-factors, novobiocin interrupted DNA-protein (6, 7) and protein-protein interactions (5). Taken together, these data indicate that novobiocin interferes with an ATP-dependent, DNA-protein or protein-
ATPase (14), acts by blocking the action of a trans-factor protein interaction essential for the formation of stable class transcription of rat or mouse rDNA have been purified to homogeneity, exhibiting ATPase activity. The RNA polymerase I1 transcription reaction at 30 °C proceeds for 30 min at 30 °C after addition of the missing component(s). Lane 1, DE-B + novobiocin → rDNA + nucleoside triphosphates (NTPs) →. Lane 2, rDNA + novobiocin → DE-B + NTPs →. Lane 3, DE-B + rDNA + novobiocin → NTPs →. Lane 4, DE-B + rDNA + NTPs → novobiocin →. The arrowhead denotes the position of the 635-nucleotide-long rDNA transcript.

FIG. 3. Effect of preincubation DE-B with novobiocin. Transcription reactions contained 240 ng of XhoI-cleaved rDNA and 15 μg of fraction DE-B. Lanes 1–4 (see below) represent the addition of novobiocin (1 mM) before, during, or after stable preinitiation complex formation. The first arrow represents a 10-min preincubation at 30 °C and the second arrow represents the 30-min transcription reaction at 30 °C after addition of the missing component(s). Lane 1, DE-B + novobiocin → rDNA + nucleoside triphosphates (NTPs) →. Lane 2, rDNA + novobiocin → DE-B + NTPs →. Lane 3, DE-B + rDNA + novobiocin → NTPs →. Lane 4, DE-B + rDNA + NTPs → novobiocin →. The arrowhead denotes the position of the 635-nucleotide-long rDNA transcript.

proteins interaction essential for the formation of stable class I, II, and III preinitiation complexes and ultimately the initiation of transcription.

Consideration was given to the possibility that the novobiocin-induced inhibition of eukaryotic gene transcription by all three RNA polymerases involved a related mechanism. We have previously suggested (3) that novobiocin, an inhibitor of ATPase (14), acts by blocking the action of a trans-factor exhibiting ATPase activity. The RNA polymerase II transcription factor TFIIE has been shown to copurify with ATPase activity (15). Similarly, Hazuda and Wu (16) have reported an ATPase (adenosinetriphosphatase, EC 3.6.1.3) activity associated with Xenopus TFIID which is involved in the novobiocin inhibition of RNA polymerase III-directed transcription (13, 14). It is unclear if an ATPase is operative in the RNA polymerase I-directed transcription of rDNA. Since none of the transcription factors involved in transcription of rat or mouse rDNA have been purified to homogeneity, the association of ATPase with these factors is at best a speculation. However, such a mechanism does not explain all the effects of novobiocin on transcription. For instance, TFIIBA is not required in the RNA polymerase III-directed transcription of tRNA genes, yet novobiocin inhibits transcription of tRNA genes (4). Further, although both TFIIB and TFIIC do not exhibit ATPase activity, novobiocin prevents their association with protein (5) and DNA (6), respectively. It therefore seems unlikely that the drug is acting through a common trans-acting factor.

Since novobiocin is a competitive inhibitor of ATP with respect to binding to DNA topoisomerase II (13) and the concentration of novobiocin necessary for inhibition of eukaryotic type II topoisomerase activity is similar to that used for inhibiting specific gene transcription (3–6), it was plausible that the novobiocin inhibition of rDNA transcription is mediated via this enzyme. However, the involvement of DNA topoisomerase II in a DNA relaxation capacity can be ruled out for the following reasons. First, linear templates were used for the transcription reaction. Second, a specific inhibitor of DNA topoisomerase II, VM-26, did not diminish transcription of this linearized gene under identical transcription conditions. In this context it should be noted that the mechanism of novobiocin action on Pol III (4) and Pol II (3) gene transcription did not involve the unwinding action of DNA topoisomerase II. However, the possibility that novobiocin alters a different, unknown, function of topoisomerase II cannot be excluded. The type I DNA topoisomerase of the archaebacterium Sulfolobus has been recently reported to contain a DNA-dependent ATPase activity (17). Such an alternative activity of DNA topoisomerase II has yet to be identified. Thus, these observations prompt us to conclude

2 L. C. Garg and S. T. Jacob, unpublished data.

FIG. 4. Effect of ATP on novobiocin inhibition of rDNA transcription. Transcription reactions contained 240 ng of XhoI-cleaved rDNA and 15 μg of fraction DE-B. Lane 1, transcription in the absence of novobiocin. Lane 2, fraction DE-B was preincubated with novobiocin (1 mM) (10 min at 30 °C) in the absence of ATP. Lanes 3 and 4, fraction DE-B was preincubated with 50 or 500 μM ATP, respectively, in the absence of novobiocin. Following preincubation, the remaining appropriate components of the transcription reactions and novobiocin (1 mM) were added and transcription proceeded for 30 min at 30 °C. The arrowhead denotes the position of the 635-nucleotide-long rDNA transcript.
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that novobiocin is a general inhibitor of eukaryotic gene transcription initiation which acts independent of the relaxing activity of DNA topoisomerase II.

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