Volume 15 Number 11 1987 Nucleic Acids Research

Novobiocin interferes with the binding of transcription factors TFIIIA and TFIIIC to the promoters of class III genes

Michael W.Van Dyke and Robert G.Roeder

Department of Biochemistry and Molecular Biology, The Rockefeller University, New York, NY 10021, USA

Received March 24, 1987; Revised and Accepted May 7, 1987

ABSTRACT

Novobiocin has been shown to inhibit class III gene transcription from both chromatin and non-chromatin templates. Since novobiocin is a well characterized inhibitor of type II DNA topoisomerases, it had been postulated that a gyrase activity is necessary for transcription. Using DNase I footprinting, we show here that novobiocin inhibits the specific binding of polymerase III transcription factors TFIIIA and TFIIIC to the promoters of the 5S RNA and VA RNA genes, respectively. Concentrations of novobiocin employed were comparable to those necessary to inhibit HeLa topoisomerase II. In vitro transcription assays, performed under equivalent conditions, demonstrated that similar novobiocin concentrations were necessary for transcription inhibition. These results strongly suggest that novobiocin interferes with transcription by inhibiting specific protein-DNA interactions.

INTRODUCTION

Novobiocin, a substituted coumarin antibiotic isolated from the species *Streptomyces*, is well documented in its ability to inhibit the function of type II DNA topoisomerases. In the case of DNA gyrase from *E. coli*, inhibition of supercoiling occurs with concentrations of novobiocin as low as 1 μg/ml (1). For eukaryotic type II topoisomerases, novobiocin concentrations in excess of 200 μg/ml are necessary in order to achieve similar levels of inhibition (2,3). For DNA gyrase, the mechanism of novobiocin action involves competition for the required binding of ATP to the B subunit of the enzyme (4). The exact mechanism for the inhibition of eukaryotic topoisomerase II by novobiocin has not been fully established.

Accurate *in vitro* transcription systems for various class III genes, including the 5S, tRNA, and virus-associated (VA) RNA genes, have been available for a number of years (5). Chromatographic fractionation of cultured cell extracts demonstrated that at least two protein fractions, termed transcription factors TFIIIB and TFIIIC, are necessary in addition to RNA polymerase III for the transcription of all class III genes (6,7). An additional protein, TFIIIA, is specifically required for 5S gene

transcription. TFIIIA binds initially to the internal promoter of the gene forming a metastable complex which is stabilized by the subsequent binding of TFIIIC (8,9). The next step in preinitiation complex formation is the rate-limiting association of TFIIIB (10), which precedes RNA polymerase III addition. In the case of the tRNA and VA genes, TFIIIC initially binds to the DNA, followed in turn by TFIIIB and RNA polymerase III (11).

Recent studies have employed novobiocin to investigate the role of DNA gyration in the establishment of transcriptionally competent chromatin templates *in vitro* and have been interpreted in terms of a direct role for DNA topoisomerase II in the RNA polymerase III-mediated specific transcription of the 5S RNA gene and polyoma DNA (12,13). Investigations with non-chromatin templates have shown a similar novobiocin-dependent inhibition of 5S RNA gene transcription *in vitro*, which was interpreted as an interference of the ATP-dependent step in stable preinitiation complex formation (14). It has been noted that all these investigations have employed concentrations of novobiocin capable of more general phenomena, for example the precipitation of arginine-rich proteins such as histones (15,16).

In this study we have used DNase I footprinting to investigate the effect of novobiocin on the sequence specific binding of transcription factors to the internal promoters of various class III genes. *In vitro* transcription reactions were also performed under identical conditions (protein, promoter, and DNA concentrations) in order to compare the effect of novobiocin on transcription. From these studies we have determined that novobiocin interferes with the site specific DNA interactions of several transcription factors. This interference may be sufficient to explain the inhibitory effect of novobiocin on the transcription of these genes.

METHODS

Transcription factors

TFIIIA from *Xenopus* oocytes was purified in the form of 7S particles according to the method of Hanas et al. (17) Partially purified TFIIIC was obtained from a Dignam-style HeLa nuclear extract (18) after chromatography over a phosphocellulose column followed by chromatography over a DEAE Sephacel column as previously described (19). The preparation of partially purified TFIIIB and RNA polymerase III employed in this study has also been previously described (19). All transcription factors used in this study were essentially free from any contaminating complementary transcription factors.

DNase I footprinting

TFIIIA interaction with the transcribed strand of the 5S RNA gene utilized the singly 3' end labeled 245 bp HindIII [α-^{32}P]/BamHI fragment containing the *X. borealis* somatic 5S RNA gene isolated from the plasmid p5S, a pUC12 subclone of the insert in plasmid pXbs201 (20). TFIIIC interaction with the transcribed strands of both the VAI and VAII RNA genes of adenovirus type 2 utilized the singly 3' end labeled 672 bp NcoI [α-^{32}P]/ApaI fragment from the plasmid pAd2. Reactions were performed in a buffer containing 20 mM HEPES (pH 7.9), 60 mM KCl, 3.5 mM $MgCl_2$, 8% glycerol, and 1 mM dithiothreitol. DNA present included 400 ng pUC12 as well as approx. 10 fmoles (10,000-20,000 cpm) labeled DNA fragment. Those reactions containing transcription factors included either 24 ng TFIIIA or 10 µg of a TFIIIC-containing fraction. Reactions were assembled on ice together with final concentrations of novobiocin as described in the figure legends, and then incubated at 30°C for 10 min in order to effect protein binding. Nuclease digestion and subsequent manipulations were performed as previously described (19).

In vitro transcriptions

Transcription reactions were performed in the aforementioned buffer with the addition of 0.6 mM each ATP, CTP, UTP, and 0.025 mM [α-^{32}P] GTP (0.7 Ci/µmole). DNA present included 380 ng pUC12 and 20 ng (6 fmoles) pAd2 or 20 ng (10 fmoles) p5S. Complementing transcription factors included TFIIIB (0.11 µg) and RNA polymerase III (0.16 µg) in quantities sufficient to insure a linear response to those same concentrations of TFIIIA and TFIIIC as employed in footprinting (see above). Reactions were assembled on ice with the addition of novobiocin to concentrations listed in the figure legend, and allowed to incubate at 30°C for 60 min before the addition of 20 µl transcription stop solution. Further processing of the samples has been previously described (19). Quantitation of transcription was performed by scintillation counting.

RESULTS

Novobiocin blocks transcription factor/promoter interactions

Figure 1 illustrates the effect of increasing novobiocin concentrations on TFIIIA binding to the 5S gene as determined by DNase I footprinting. The reactions were performed with sufficient TFIIIA to render its well-characterized protection pattern (21). Addition of increasing amounts of novobiocin exhibited no effect on this pattern until a final concentration of

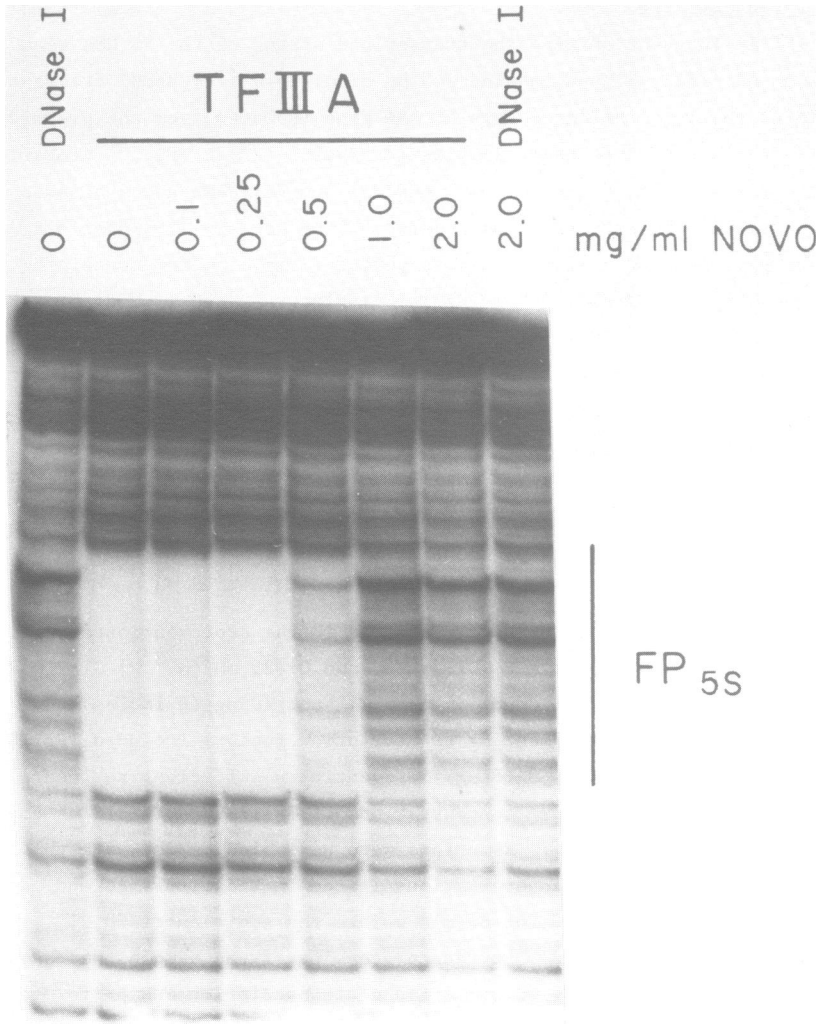

Figure 1:
Inhibition by novobiocin of TFIIIA binding to the 5S RNA gene. DNase I footprinting reactions were performed in the presence (TFIIIA) or absence (DNase I) of 24 ng TFIIIA and final concentrations of novobiocin as indicated above each lane. The location and extent of the TFIIIA footprint on the 5S gene is indicated to the right of the figure.

0.5 mg/ml was achieved, at which point a partially protected TFIIIA footprint was observed. No qualitative changes in either the protected region or the adjacent hypersensitive cleavage sites were observed, changes which would be

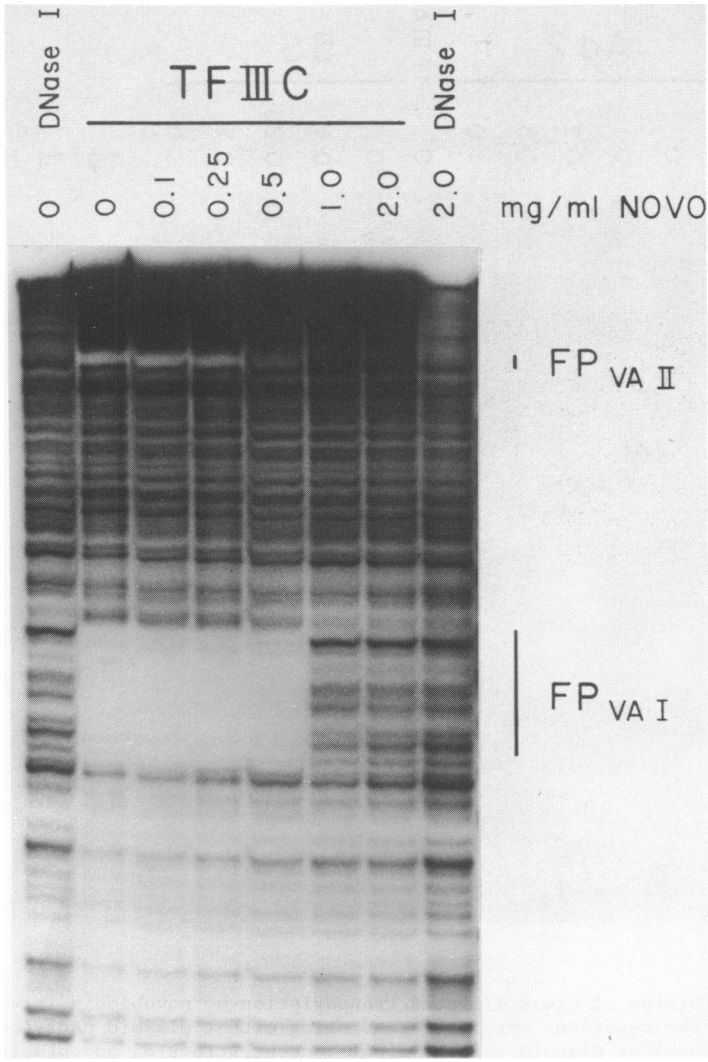

Figure 2:
Inhibition by novobiocin of TFIIIC binding to the VA genes. DNase I footprinting reactions were performed either in the presence (TFIIIC) or absence (DNase I) of 10 µg of a TFIIIC-containing fraction and final concentrations of novobiocin as indicated. The locations of the TFIIIC footprints on both the VAI and VAII genes is indicated to the right of the figure.

indicative of alterations in the TFIIIA-5S DNA interaction. Also note that the highest concentrations (2.0 mg/ml) of novobiocin did not change either the DNase I cleavage rate or specificity; therefore, DNase I may be considered a

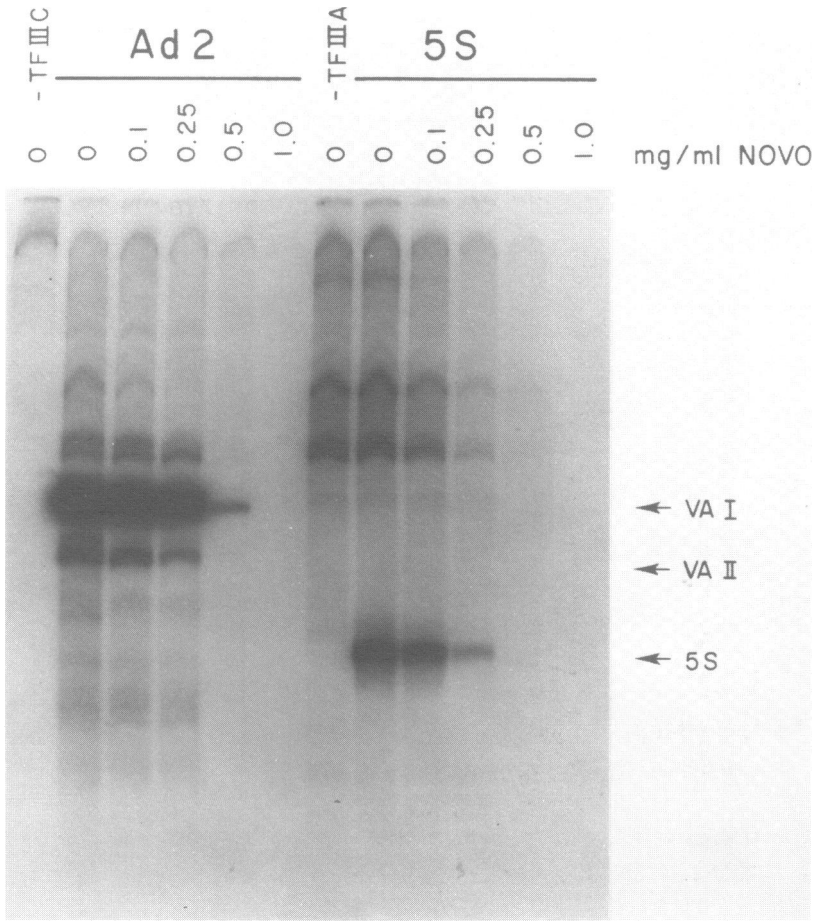

Figure 3:
Inhibition of class III gene transcription by novobiocin. In vitro transcription reactions were performed using either plasmid pAd2 (containing both VA genes) or plasmid p5S and final concentrations of novobiocin as indicated. Control reactions were deficient in one transcription factor (-TFIIIC for pAd2, -TFIIIA for p5S). RNA products are identified to right of figure.

suitable probe for investigating protein-DNA interactions in the presence of novobiocin. From these data it would appear that fairly low concentrations of novobiocin are capable of preventing the binding of TFIIIA to the internal promoter of the 5S RNA gene without appreciably altering the normal form of TFIIIA-5S DNA interactions.

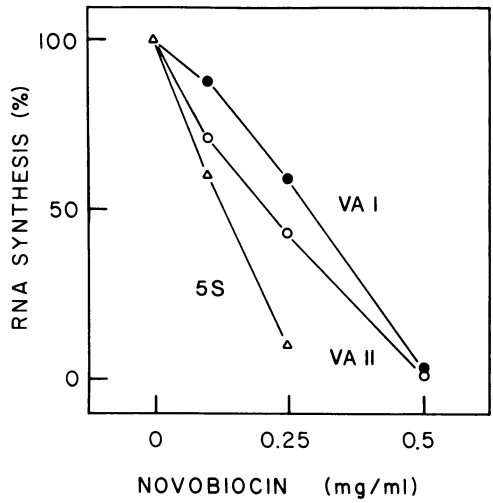

Figure 4:
Quantitation of transcription inhibition by novobiocin. Transcription from figure 3 is presented as a percentage of maximal RNA synthesis (novobiocin minus reaction) versus novobiocin concentration. Closed circles are for VAI, open circles are for VAII, and open triangles are for 5S gene transcription. 100% RNA synthesis corresponded to 73, 7, and 20 fmoles RNA from the VAI, VAII, and 5S genes, respectively.

Figure 2 illustrates the effect of novobiocin on the DNase I footprints observed with partially purified TFIIIC and both the VAI and VAII RNA genes of adenovirus type 2 (11,19). Increasing concentrations of novobiocin were capable of preventing TFIIIC binding to the B-block consensus elements in both of these genes; concentrations of novobiocin necessary to achieve 50% loss of protection were 0.5 and between 0.5-1.0 mg/ml for the VAII and VAI genes, respectively. This difference in novobiocin sensitivity for the two VA genes could reflect the relative affinities of TFIIIC for the two genes (19). As in the case of the TFIIIA-5S DNA interaction, no alterations in the standard TFIIIC-VA DNA interactions, indicated by both protections and hypersensitivities, were observed with intermediate concentrations of novobiocin. Thus the interference by novobiocin of the TFIIIC-VA DNA interaction appears analogous to that observed for TFIIIA-5S DNA, i.e. a loss of binding without any pertubation in the overall form of interaction.

Interestingly the concentration of novobiocin necessary to observe a 50% loss of TFIIIA or TFIIIC footprinting appeared to be proportional to the concentration of transcription factor present in the reaction (data not

shown). This is consistent with a model in which novobiocin interacts directly with transcription factors, and that these protein-novobiocin complexes are then incapable of binding to DNA.

Novobiocin interferes with *in vitro* transcription

In vitro reconstituted transcription reactions were performed under conditions of limiting genes and saturating transcription factors analogous to those employed in DNase I footprinting. This allowed us to make a direct comparison between the concentrations of novobiocin necessary to prevent factor binding to DNA, the initial step in stable preinitiation complex formation, and those necessary to inhibit transcription. As shown in figure 3, the complementary proteins alone were insufficient to reconstitute specific transcription from the 5S RNA or either of the two VA RNA genes. Addition of quantities of either TFIIIC (VA gene transcription) or TFIIIA and TFIIIC (5S gene transcription) sufficient for footprinting resulted in high levels of transcription from each of the three genes. Increasing concentrations of novobiocin resulted in transcription inhibition of all three genes. Quantitation of these data (figure 4) revealed that the novobiocin sensitivity of the three genes was different, with 5S gene transcription being the most sensitive and VAI gene transcription being the least sensitive. It is noteworthy that this order of sensitivity to novobiocin is identical to that found by the footprinting assays, though the amount of novobiocin necessary to observe 50% inhibition of transcription was roughly 1/3 that necessary to observe 50% loss of the initial transcription factor binding to each gene.

DISCUSSION

Novobiocin, an inhibitor of eukaryotic type II topoisomerase activity, has been shown by several investigators to inhibit class III gene transcription at concentrations comparable to those which inhibit topoisomerase activity (12,13,14). This observation has been used to argue for a direct involvement of topoisomerase II in the formation of transcription complexes. Our investigations using DNase I footprinting and partially purified transcription factors demonstrated that the initial protein-DNA interactions between transcription factors and the internal promoters of class III genes are themselves inhibited by concentrations of novobiocin comparable to those used to inhibit eukaryotic topoisomerase II.

Does the loss of initial transcription factor binding to class III genes fully account for the observed sensitivities of transcription to novobiocin? Comparisons between transcription assays and DNase I footprinting indicated

that RNA synthesis from each of the three genes was somewhat more sensitive to novobiocin than the corresponding transcription factor-promoter interactions. It is should be noted that the transcription sensitivities were different for each gene, this precluding the involvement of a single common factor or step in stable complex formation possessing the sole sensitivity to novobiocin. Rather, the differences may reflect not only the protein-DNA interactions of the initial transcription factors, but also the summation of novobiocin sensitivities for the various protein-DNA (and perhaps protein-protein) interactions necessary for stable complex formation and subsequent transcription. Evidence for this hypothesis was recently demonstrated by Felts et al.(22). Using an *in vitro* yeast transcription system, they found that novobiocin specifically inhibited the interactions between TFIIIB and the other transcription factors on the 5S RNA gene.

The nonspecific inhibition of protein-DNA interactions by novobiocin can be considered a general characteristic of this molecule. In addition to the interactions described in this paper (TFIIIA-5S, TFIIIC-VA), we have also observed the novobiocin-dependent inhibition of both nuclear factor I binding to the adenovirus origin of replication (23) and the binding of the upstream stimulatory factor to the adenovirus major late promoter (M. Sawadogo, unpublished observations)(24). Typically, the inhibition of binding by novobiocin appears dependent on protein concentration, and reflects the relative affinity a protein has for a particular DNA sequence. Thus any experiments involving protein-DNA interactions and novobiocin concentrations greater than 100 µg/ml should take into consideration these effects before proceeding to any more involved interpretations.

ACKNOWLEDGEMENTS

We thank Paul Martin for providing partially purified TFIIIB and Michele Sawadogo for her critical reading of the manuscript. This research was supported by Public Health Service grants CA 42567 and CA 34223 from the National Institutes of Health to R.G.R and with general support from the Pew Trusts. M.W.V. was supported by a postdoctoral fellowship (GM 09949) from the National Institutes of Health.

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