The 5.8S ribosomal RNA gene of *Trypanosoma brucei*: structural and transcriptional studies

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ABSTRACT

To further investigate the process of discontinuous transcription in trypanosomes, the 5.8S rRNA gene, present in the trypanosome genome as part of the multicopy rRNA gene cluster, has been cloned, sequenced, chromosomally mapped, and used in transcriptional studies. The gene's sequence confirms its identity and indicates that it is less conserved evolutionarily than the trypanosome 5S rRNA gene previously described by our laboratory (6). Examination of the chromosomal locations of the gene by pulsed-field gradient gel electrophoresis shows that the 5.8S rRNA genes occur on at least four differently-sized chromosomes in *T. b. rhodesiense* and at least three differently-sized chromosomes in *T. b. brucei*. The 5.8S transcript was analyzed by a run-off transcription assay using isolated nuclei. These studies strongly suggest that the 5.8S rRNA gene is transcribed as part of a larger precursor rRNA independently of the spliced leader RNA and by a different RNA polymerase than either the spliced leader or 5S rRNA transcripts. Transcription of the trypanosome 5.8S rRNA is insensitive to very high levels of alpha-amanitin, a feature of the 5.8S rRNA in higher eukaryotes.

INTRODUCTION

Gene transcription in African trypanosomes, unicellular parasites of humans and other mammals, differs considerably in some respects from transcription in other eukaryotes. Trypanosome transcription appears to be discontinuous for many protein-coding genes, i.e., the mature transcripts are composites of transcripts derived from two unlinked loci (1,2). In all cases studied, a conserved 35 nucleotide, nontranslated sequence at the 5'-termini of the composite mRNAs is transcribed from a 1.35 kb DNA repeat unit present in about 200 copies in one or more tandem arrays in the genome (3,4,5). The rest of the composite transcript is derived from independent coding regions elsewhere in the genome. The 35 nucleotide sequence, called the spliced leader or mini-exon, is probably not present on all trypanosome transcripts. For

example, we recently demonstrated that the spliced leader does not participate in the transcription of the trypanosome 5S rRNA gene (6).

Another unusual feature of transcription in at least some trypanosome species is the finding of only a single peak of RNA polymerase activity using standard chromatographic techniques (7; T. Weil, University of Iowa, personal communication). Eukaryotes typically contain three DNA-dependent RNA polymerases (RNAPs) which can be resolved by DEAE-Sephadex chromatography (9): RNAP I which transcribes the large rRNA precursors, RNAP II which transcribes mRNAs, and RNAP III which transcribes 5S rRNA and tRNAs. It is not clear whether the finding of a single peak of RNAP activity in trypanosomes indicates the absence of multiple classes of RNAP. The fact that discontinuous transcription apparently does not occur in 5S rRNA synthesis and that the gene has a typical RNAP III transcription control region (6) suggests that trypanosomes have more than one type of RNAP. Recent findings that synthesis of various trypanosome transcripts is differentially sensitive to alpha-amanitin (8; D.M.D., M.J.L. and J.E.D., manuscript submitted) also suggest the presence of multiple classes of RNAP in trypanosomes. To further characterize transcription in trypanosomes, we have cloned and sequenced the 5.8S rRNA gene from *T.b.brucei*, and have studied its transcript, as an example of a gene transcribed by RNAP I in other eukaryotes (9).

MATERIALS AND METHODS

Trypanosomes of *Trypanosoma brucei brucei* clone IaTat 1.2, MiTat 1.8b, and VAT D.20 (a relapse population of MiTat 1.6) and *Trypanosoma brucei rhodesiense* clone WRATat 1.1 were grown and RNA and DNA isolated as previously described (10).

Cloning the 5.8S rRNA gene from *T.b.brucei* genomic restriction fragments ligated into the plasmid pUC 9 was performed using methods previously described (5). A synthetic 21-mer oligonucleotide complementary to a portion of a partial trypanosome 5.8S rRNA sequence reported by others (11), and shown to be nucleotide positions 8-28 of the 5.8S rRNA in this manuscript, was used to screen the library of cloned genomic DNA fragments under conditions previously described (5). The oligonucleotide was synthesized by Dr. Joseph Walder of the Molecular Biology Core of the

Diabetes and Endocrine Research Center, University of Iowa, using a Beckman System 1 DNA Synthesizer.

Genomic Southern blots and DNA sequence analysis of the trypanosome 5.8S rRNA gene were performed using methods previously described (5). Computer analysis of DNA sequence data was performed with an IBM-PC/XT using the PCS DNA sequence analysis programs (12). Primer extension analysis of trypanosome 5.8S rRNA, using the oligonucleotide described above, was performed as previously described (6, 13).

Pulsed field gradient (PFG) gel electrophoresis of trypanosome DNA was essentially performed as described by Schwartz and Cantor (14) and Van der Ploeg et al. (15). Electrophoresis of DNA samples was for 24 hours at 15 °C with 15 V/cm North/South and 5 V/cm East/West at a pulse frequency of 2 min. DNAs were blotted to nitrocellulose and probed with the nick-translated (5), cloned trypanosome 5S rRNA gene (6) and 5.8S rRNA gene (this paper).

Transcription studies were performed using isolated trypanosome nuclei and [α -³²P]UTP (D.M.D., M.J.L. and J.E.D., manuscript submitted) under conditions previously described (16), and the labeled RNA was hybridized to nitrocellulose-bound DNA using conditions previously described (17,18).

RESULTS

Cloning and DNA sequence determination of the 5.8S ribosomal RNA gene

Trypanosomes possess six small ribosomal RNAs which have been partially characterized (11). The third largest of these, was tentatively identified as the 5.8S rRNA on the basis of RNA sequence analysis of its 5'-terminus (11). This RNA species, when end-labeled, hybridizes to an approximately 2 kilobase Alu I restriction fragment (11). We prepared a partial genomic library in the plasmid pUC 9 using 1200-4000 base pair Alu I genomic restriction fragments from T.b.brucei DNA. A synthetic 21-mer oligonucleotide which was complementary to a portion of the partial 5.8S rRNA sequence (11) and is shown in Figure 1B, was used to screen the library. Numerous clones hybridized to the oligonucleotide probe and one, plA2-5.8S, was selected for further study.

Figure 1. Restriction map and partial DNA sequence of the 2.4 kb Alu I restriction fragment, cloned into plasmid pUC 9, containing a 5.8S rRNA gene. (A) Restriction map showing the DNA sequencing strategy; solid line indicates the trypanosome insert in plasmid pUC 9; thicker portion indicates 5.8S gene; dotted lines indicate vector sequence. Restriction enzymes: H = Hind III (from vector polylinker sequence), R = Eco RI (within trypanosome insert and also at the vector polylinker), F = Hinf I and X = Xho I. (B) DNA sequence of a portion of the 2.4 kb trypanosome insert containing the 5.8S rRNA gene. The gene sequence (determined by consensus with other 5.8S rRNA sequences) is underscored. The synthetic oligonucleotide complementary to positions 8-28 of the gene sequence is also shown.

Figure 2. Summary of gene sequences of 5.8S rRNAs. All sequences were predicted from the rRNA sequences in Erdmann et al. (19) except the T.b.brucei sequence (this paper). Also shown are divergence values, Knuc, of each species compared with the T.b.brucei sequence derived according to the method of Ohama et al. (21). Asterisks indicate exact matches among all sequences. Plus signs indicate exact matches between the T.b.brucei and Crithidia fasciculata sequences.

A partial restriction map of the 2.4 kb insert in plasmid pLA2-5.8S is shown in Figure 1A. Southern blots using the oligonucleotide (not shown) localized the hybridizing region to near

the middle of the insert. This region was subjected to Maxam-Gilbert DNA sequencing by the strategy shown in Figure 1A. The sequence is given in Figure 1B. It contains a region of 170 nucleotides (underscored in the figure), which is the correct size for a 5.8S rRNA coding region and which agrees well with the partial RNA sequence of the trypanosome 5.8S rRNA (11). As shown in Figure 2, a comparison of the sequence with a variety of other 5.8S rRNA derived DNA sequences revealed significant homology. The trypanosome 5.8S rRNA coding region is especially closely related (sharing 89% overall nucleotide matches) to the complete 5.8S rRNA sequence of *Crithidia fasciculata*, an insect trypanosome (19). Based on the conservation of size and sequence with other 5.8S rRNAs and a variety of other evidence presented below, we conclude the region sequenced contains the trypanosome 5.8S rRNA gene. A further confirmation of the identity of the 5.8S rRNA gene clone is provided by the result of RNA dot blots which show hybridization of the clone p1A2-5.8S to gel purified trypanosome 5.8S rRNA (data not shown). Since this specific cloned gene has not been unambiguously demonstrated to be transcriptionally active, there is a possibility that it is a pseudogene. However, since the gene is highly homologous to the 5.8S rRNA, it is adequate for the subsequent hybridization studies.

Another feature to emerge from the sequence comparison between species is the strong conservation of nucleotides 45-56 among the sequences presented. In our examination of a recent compilation of 29 5.8S rRNA sequences (19), this region was found to have the highest sequence conservation. Therefore, this sequence may have a functional significance which is as yet undefined. Conversely, almost no sequence conservation occurs in the 3' 60 nucleotides of the 5.8S rRNA derived sequences, which may be related to the postulated base pairing interaction between 5.8S and 28S rRNAs in the ribosome (20).

To determine the evolutionary relationship of the trypanosome 5.8S rRNA sequence with those of other species, Knuc values for the evolutionary divergence of the 5.8S rRNA sequence among species were calculated by the method of Ohama et al. (21). As noted above, the trypanosome sequence is most closely related to the *Crithidia fasciculata* sequence. In fact, to a first approximation, the 5.8S sequences of these two organisms are more

closely related ($\text{Knuc} = 0.13$) than are their 5S rRNA sequences ($\text{Knuc} = 0.25$), according to our recent analysis (6). In general, however, the trypanosome 5S rRNA sequence is more conserved evolutionarily than is the 5.8S rRNA sequence when the sequences are compared to those in distantly related species. We observe a striking divergence in the 5.8S rRNA sequence between the ciliated protozoan, Tetrahymena thermophila ($\text{Knuc} = 0.9$) and the trypanosome (a non-ciliated protozoan), which was also true in comparing the 5S rRNA sequences (6). This exceeds the values for a comparison with even such distantly-related species as Homo sapiens.

Chromosomal location of ribosomal RNA genes in trypanosomes

Previous studies by one of us have shown that a clone containing a portion of the large ribosomal repeat hybridizes predominantly to the chromosome-sized DNA molecules that do not migrate from the gel slot and to those of 2 megabases (Mb), for a variety of kinetoplastid species (22). We used PFG gels to compare the chromosomal location of the 5.8S rRNA gene in both T.b.brucei and T.b.rhodesiense to the chromosomal location of the 5S rRNA gene. The analysis of chromosome-sized DNA in these gels allows separation of the 2 Mb region which was not possible previously (see Figure 3). Plasmid pIA2-5.8S hybridizes predominantly to DNA which remains in the slot and to chromosomes in the size range of 2 Mb for both T.b.brucei and T.b.rhodesiense. We are able to distinguish at least three loci for T.b.brucei and at least four loci for T.b.rhodesiense (Figure 3, panel 4). For T.b.rhodesiense, the relative hybridization intensities are 70-80%, 1-2%, 20-30%, and 1-2% from the largest to the smallest hybridizing species, respectively, as determined by densitometric scanning. Control hybridizations with VSG gene probes show that the banding pattern is specific and does not result from degradation of DNA molecules. As is shown in Figure 3, panels 1 and 2, the repeat element which contains the 5S rRNA coding region appears to be restricted to the very largest DNA species which remain at the slot. Therefore, the repeat elements bearing the 5.8S rRNA gene sequence which are located on the 2 Mb chromosomes are not linked in the genome with the repeats that bear the 5S rRNA gene. More detailed analysis of conventional Southern blots and restriction mapping of phage clones does not give evidence of

linkage between the tandem array of 5S rRNA gene repeats and that of the large rRNA repeat (data not shown).

Analysis of the 5.8S gene transcript

To compare trypanosome transcription with that of other species, we used the synthetic oligonucleotide and cloned gene to investigate the transcript of the 5.8S rRNA gene. In particular, we sought to determine whether the 5.8S transcript had the 35 nucleotide spliced leader sequence at its 5'-terminus, i.e. whether it was discontinuously transcribed. For this purpose, a primer extension experiment was carried out. The 21-mer oligo-

Figure 3. Chromosomal locations of the 5S and 5.8S gene in *Trypanosoma b. brucei* and *Trypanosoma b. rhodesiense*. The autoradiographs in panels 2 and 4 are prepared from blots of the ethidium bromide stained agarose gels shown in panels 1 and 3, respectively, which resulted from PFG gel electrophoresis of trypanosome DNAs. These were: *T.b.brucei* VAT D.20 DNA (lanes A,C and E of panels 1 and 2 and in lane A of panels 3 and 4), *T.b.brucei* MiTat 1.8b DNA (lane G of panels 1 and 2), and *T.b.rhodesiense* WRATat 1.1 DNA (lanes B,D and F of panels 1 and 2 and lanes B and C of panels 3 and 4). Panel 2 was hybridized with the cloned 5S gene (6) and panel 4 was hybridized with the cloned 5.8S gene insert (this paper). Brackets indicate the area in which the 2 Mb fraction of chromosome-sized DNA is separated. Arrowheads show chromosomes which hybridize to a minor extent with the 5.8S probe. Bending of the lanes in panels 3 and 4 was due to local gradient field and migration artifacts (22).

nucleotide (see Figure 1B) was end-labeled and used in the extension analyses shown in Figure 4. Lane a shows a DNA sequencing ladder used as a size standard. Lanes b and c show primer extension assays using nuclear and total trypanosome RNA, respectively. These show that the primer is extended 7 nucleotides to the predicted end of the 5.8S rRNA template as shown in Figure 2. Lane d is a control experiment using yeast RNA and

Figure 4. Primer extension analysis of the 5.8S rRNA transcript. Autoradiogram of 10% polyacrylamide electrophoresis gel of primer extension analysis. Lane a: DNA sequencing ladder for size markers; lanes b and c: primer extension using the 21-mer complementary to the 5' end of the trypanosome 5.8S rRNA using 6 ug of nuclear RNA and 6 ug of total RNA from trypanosomes, respectively; lane d: primer extension using 6 ug of Saccharomyces cerevisiae RNA. Lane e uses a 30-mer complementary to a portion of the trypanosome spliced leader (5) and 6 ug of total trypanosome RNA. Numbers 21 and 30 indicate lengths, in nucleotides, of the oligonucleotide primers used. Note that the 21-mer is extended 7 nucleotides in lanes b and c and the 30-mer is extended 5 nucleotides in lane e. A probable 22-mer contaminant in lanes b-d is extended 7 nucleotides in lanes b and c.

reveals no extension of the primer. Evident in each lane containing the 21-mer oligonucleotide (b,c,d) is an additional labeled species which is 22 nucleotides in length. This species is also extended 7 nucleotides and appears to be a contaminant in

Figure 5. Comparison of transcription of the 5S and spliced leader genes and their flanking regions. (Left) Panel 1: ethidium stained agarose gel of a) Eco RI/Hind III digest of pIA2-5.8S and b) Acc I/Hind III digest of a plasmid containing the mini-exon repeat (5). Panels 2 and 3 are autoradiograms of the hybridization of nascent transcripts synthesized by nuclei in the presence of [α - 32 P]UTP and 0 or 1000 ug/ml alpha-amanitin, respectively. Black hatch marks on the left show the positions of Hind III digested lambda DNA standards which are, in descending order of size, 23.1 kb, 9.4 kb, 6.6 kb, 4.4 kb, 2.3 kb, 2.0 kb and 0.6 kb. (Right) Maps showing expected fragments from the restriction digests; in both cases, a 2.7 kb band derived from the pUC 9 cloning vector is also expected and is visible in Panel 1. Abbreviations: A= Acc I, H = Hind III and R = Eco RI.

the oligonucleotide preparation bearing an additional nucleotide at the 5'-terminus. This experiment also reveals no evidence of a stable 5.8S rRNA species bearing an extension consisting of the 35 nucleotide conserved spliced leader sequence. A control experiment, shown in lane e, is a primer extension of a 30-mer complementary to the 5' end of the trypanosome spliced leader (5) extended against total trypanosome RNA. The primer is extended 5 nucleotides, in agreement with an earlier determination of the 5'-terminus of the mini-exon transcript (18).

A second experiment is shown in Figure 5. Transcripts

labeled in a nuclear run-off assay were used to determine if regions adjacent to the rRNA gene are actively transcribed and to assess the alpha-amanitin sensitivity of this transcription (16-18) in comparison with that of the spliced leader coding region. A Hind III/Eco RI double digest of p1A2-5.8S was electrophoresed through an agarose gel and blotted to nitrocellulose. The digest produces 2 insert fragments, the larger of which (1500 bp) contains the 5.8S gene (see Figure 1A). Lane 2a of Figure 5, an autoradiogram of the same DNA hybridized to labeled transcripts made in the presence of no alpha-amanitin, shows that the entire cloned region is actively transcribed. Similar blots using a variety of restriction digests of the 2.4 kb restriction fragment containing the rRNA gene gave the same results (data not shown), suggesting that transcription occurs throughout the region of the 5.8S rRNA gene, as expected for a eukaryotic large rRNA gene array. This is in contrast to the result for the spliced leader gene repeat (lane 2b) in which nascent transcripts bind only to the restriction fragment which encodes the spliced leader transcript. Lane 3a shows that in the presence of 1 mg/ml alpha-amanitin, sequences on both 5.8S rRNA insert fragments continue to be transcribed. No transcripts which hybridize to the spliced leader sequence are synthesized at this concentration of alpha-amanitin (lane 3b). This suggests that the mini-exon and the 5.8S gene clusters are transcribed by different RNA polymerases. The 5.8S rRNA may be synthesized by a trypanosome RNAP I equivalent since that enzyme is typically insensitive to high concentrations of alpha-amanitin (9). As a control, single-stranded probes were generated for the 5.8S gene in bacteriophage M13 and hybridized to nascent transcripts. These experiments demonstrate that transcription is specific for the coding strand (i.e. transcription is asymmetric and specific in the nuclear transcription system) for the 5.8S rRNA and mini-exon coding regions, as well as several other trypanosome genes (data not shown).

Previously, we have shown that the mature 5.8S species is not capped *in vitro* by vaccinia virus guanylyltransferase (23) either before or after periodate treatment (6; M.J.L., D.M.D., and J.E.D., manuscript submitted). This suggests its 5' terminus bears a monophosphate. The simplest interpretation of this data

together with the above findings is that the 5.8S species is synthesized as part of a larger precursor transcript which subsequently undergoes processing to produce the individual, mature rRNA species.

DISCUSSION

Previous studies of transcription in trypanosomes revealed two features which have no corollaries in other organisms. First, there is a conserved 35-nucleotide spliced leader on the 5' end of many, if not all, protein-coding transcripts. Second, distinct peaks of RNA polymerases could not be distinguished by standard chromatographic techniques (7; T. Weil, University of Iowa, personal communication). This introduced the possibility of a primitive, single polymerase transcription system for *Trypanosoma* species. Therefore, we undertook a detailed analysis of transcription in trypanosomes. Our approach has been to investigate the transcription of genes which in other species have been clearly shown to be synthesized by distinct RNA polymerases with different alpha-amanitin sensitivities. Several lines of evidence support the conclusion that the cloned sequence described here codes for the trypanosome 5.8S rRNA. A comparison of its sequence to that of 5.8S rRNA derived sequences of other species, particularly that of *Crithidia fasciculata*, reveals significant homologies in length and sequence. The 5' end of the 5.8S rRNA deduced from the sequence of the clone was confirmed by primer extension of the synthetic oligonucleotide. The clone hybridized strongly in a RNA dot blot to gel-purified small rRNA 3 which had been tentatively identified as the trypanosome 5.8S rRNA (11). Finally, the clone can hybrid select transcripts whose synthesis is resistant to very high levels of alpha-amanitin and which appear to have a monophosphate at the 5' end--both properties expected for the 5.8S transcript.

We have used the cloned gene to study the genomic organization of 5.8S genes in the genomes of *T.b.brucei* and *T.b.rhodesiense*. We detected a clear difference in the banding pattern in the 2 Mb region between *T.b.brucei* and *T.b.rhodesiense* indicating a difference in the chromosomal organization of the 5.8S genes and, likely, the entire large ribosomal repeat. This variability

between T.b.brucei and T.b.rhodesiense (which can be considered the same species (6)), suggests that large chromosomes are involved in chromosomal recombination events altering their composition. Previously it has been shown that recombination events among the small chromosomes (50 to 200 kb) occur frequently and may be associated with antigenic variation (15). The variation in chromosomal distribution of 5.8S genes suggests chromosomal recombinations may occur infrequently in the larger chromosomes as well.

Hybridization to Southern blots shows that the 5.8S gene is part of a tandem repeat unit about 20 kb in size (data not shown). Our analysis of the chromosomal locations of the 5.8S and 5S rRNA genes reveals that their genomic loci have distinct patterns of size distribution and the 5.8S rRNA genes which reside on 2 Mb chromosome-sized DNAs are not closely linked to 5S genes. Probably there is no linkage between the repeats encoding the 5S and 5.8S rRNA genes since no overlap in the cloned portions of the long tandem arrays for each gene can be found (L.V.R., unpublished results). Further characterization of the flanking regions of each gene's tandem array should answer this question. Since it is not yet possible to resolve distinct size classes within the large DNA molecules which stay in the slot of the gel, we are unable to determine whether the 5S gene also occurs in more than one tandem array.

Since the 5S rRNA is transcribed as a spliced leader-independent species (6), we investigated whether the synthesis of the 5.8S rRNA is also independent of the spliced leader. The results of the primer extension analysis do not reveal an RNA species related to the 5.8S rRNA which bears the spliced leader sequence. In addition, transcription of the spliced leader sequence is sensitive to high levels of alpha-amanitin (Figure 5, lane 3b) and no diminution of nascent transcripts hybridizing to the 5.8S clone occurs at similar levels of alpha-amanitin. Therefore, it is unlikely that the production of 5.8S rRNA depends on transcription by the polymerase that transcribes the spliced leader sequence, which might be predicted from a "jumping polymerase" model (18). Unlike the 5S rRNA which could be clearly shown to have a 5' polyphosphate terminus, our capping experi-

ments suggest that the 5.8S rRNA most likely has a 5' monophosphate terminus (see above). Therefore we cannot completely exclude the possibility that a very unstable 5.8S-related species bearing the spliced leader sequence occurs. Because of the arguments made above, however, we believe this possibility to be very unlikely. This indicates that the bimolecular splicing or priming event which places the spliced leader sequence at the 5' end of protein-coding genes is not required for all transcription in the organism. Since at least some trypanosome RNA polymerases do not require a 35 nucleotide primer to carry out transcription and consensus splice sites occur at the junction of the leader sequence and the body of trypanosome mRNAs, the priming model of transcription seems unlikely. This may be contrasted to viruses such as influenza in which utilization of a primer seems to be a requirement for all transcription of the viral genome (25).

Transcription of the 5.8S rRNA is insensitive to very high levels of alpha-amanitin. Transcription of regions flanking the 5.8S coding region is also alpha-amanitin insensitive. This is consistent with a model in which the 5.8S gene is transcribed as a precursor with the large ribosomal RNAs which is subsequently processed to yield the individual 28S, 18S, and 5.8S rRNAs as well as up to four other small rRNAs. Supporting this model are the restriction maps of plasmids by Hasan et al. (11) which localize the 5.8S rRNA gene to a region between the 18S and 28S coding regions, similar to its location in other species, where these rRNAs all form part of a single transcription unit (23). Since our studies suggest the 5.8S rRNA has a 5' monophosphate, we conclude that the 5.8S rRNA is cleaved from a larger precursor RNA as Hasan et al. (11) have predicted. While this manuscript was in preparation, Kooter and Borst (8) reported that nascent transcripts synthesized in isolated nuclei which are insensitive to high levels of alpha-amanitin bind to an 18 kb fragment which contains a major part of the large ribosomal repeat. While these studies did not distinguish the transcripts hybridizing to the 5.8S gene from other regions of the repeat, their results are consistent with our findings.

In summary, our studies indicate that the 5.8S rRNA, and probably the entire large ribosomal RNA precursor, is synthesized

and functions in the cell independently of the spliced leader. Combining these results with our previous investigations on the 5S rRNA gene, we conclude that the spliced leader sequence is only involved in the function and/or synthesis of protein-coding genes which in other eukaryotes are synthesized by RNAP II.

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Abbreviations: bp, base pair; kb, kilobase; rRNA, ribosomal RNA; RNAP, RNA polymerase.

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