Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 2137–2141, April 1984
Genetics

In vitro mutagenesis and transcriptional analysis of a mouse ribosomal promoter element

(RNA polymerase I promoter/bisulfite mutagenesis/transcription *in vitro*)

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Communicated by M. Lindauer, December 13, 1983

ABSTRACT **An RNA polymerase I control region essential for initiation of pre-rRNA transcription has been identified by mutagenesis *in vitro* of mouse rDNA (ribosomal RNA genes) and transcription in a cell-free system derived from Ehrlich ascites cells. Substitution of nucleotides between −35 and −14 by foreign DNA sequences caused a loss of template activity, which indicates that an important promoter element is located within this region. To identify the nucleotides essential for RNA polymerase I function, single and multiple point mutations within this control region were generated and the modified DNAs were assayed for template activity. The phenotypes of mutants in which C-to-T transitions have been introduced at positions −36, −31, −27, −22, −21, and −13 were identical to the wild type. Conversion of G to A at position −15 resulted in a 20% increase of promoter activity, whereas a G-to-A transition at −16 decreased transcription by 95%. Competition experiments between mutant and wild-type DNAs suggest that the guanine at −16, which is evolutionarily highly conserved, interacts with essential components of the transcription apparatus.**

The DNA sequences controlling the expression of eukaryotic genes transcribed by RNA polymerases II and III have been recently defined by methods of reverse genetics (1–6). These methods involve the introduction, *in vitro*, of deletions, substitutions, or point mutations into cloned DNA and the subsequent assay of their effects on the biological activity of the DNA.

As yet, little is known about the DNA sequences constituting the RNA polymerase I promoter. Analyses of the template activity of mutants with deletions in the rRNA genes (rDNA) have demonstrated that a major component of this promoter lies upstream of the transcription initiation site (7–9). For mouse rDNA we have shown that 39 nucleotides preceding the start site contain all the sequence information required for efficient, accurate transcription initiation (7). Deletion of five more bases up to position −34 results in a reduction to 1/10th in template activity, while removal of all but 12 nucleotides from the 5′ flanking region causes a loss of transcriptional competence. This finding suggests that control sequences lie within the region −12 to −39. However, interpretation of results obtained from deletion mutants is complicated by the fact that essential regions have been removed, thus bringing other sequences abnormally close. Therefore, it is not possible to distinguish between the effects on promoter activity of (*i*) the deletion and (*ii*) bringing upstream sequences nearer to the gene. To overcome this difficulty we have constructed a series of mutants containing deletions, substitutions, or base changes within the control region. Using a cell-free transcription system, which allows accurate transcription of mouse rDNA by RNA polymerase I, we have compared the transcriptional efficiencies of wild-type and mutant DNA templates. Functional analyses of these mutants reveal the importance of a guanine at position −16 for promoter function *in vitro*.

MATERIALS AND METHODS

Construction of the Deletion/Substitution Mutant pMrΔ-14-35. To construct the mutant pMrΔ-14 the termini of a *Bst*NI fragment, derived from pMrSP (10), containing mouse rDNA sequences from −12 to +292 were filled in by the Klenow fragment of DNA polymerase and the fragment was inserted into the *Sma* I site of pUC9. The resulting cloned DNA was linearized with *Sal* I. After the sticky ends had been filled up with DNA polymerase, this linearized plasmid was cleaved with *Hin*dIII and used as a vector. The clone pNTS-35-169 containing upstream regions from −35 to −169 was constructed by inserting a *Sau*3A fragment (extending from position −169 to −39) in the rDNA into the *Bam*HI site of pUC9. This plasmid was then cleaved with *Hin*dIII and *Sma* I and the resulting 325-base-pair (bp) fragment was ligated into the pMrΔ-14 vector to yield the mutant pMrΔ-14-35 (Fig. 1*A*). Thus pMrΔ-14-35 consists of mouse rDNA from −169 to +292 with a deletion of 22 bases between positions −14 and −35. Between these two positions lies a 14-bp insert of pUC9 sequences including a unique *Bam*HI site used in construction of the spacing mutants pMr-14BS-$35_{1,\ 2,\ \text{and}\ 3}$.

Construction of Point Mutants. Base substitution mutants were constructed as described by Everett and Chambon (11). For this the *Sal* I/*Sma* I fragment from the mutant clone pMrΔ-14-35 and from the wild-type pMrWT were cloned in bacteriophage M13 vectors, resulting in M13 MrΔ-14-35 and M13 MrWT. Both M13 vectors mp8 and mp9 were used to obtain the inserts in both possible orientations. The double-stranded replicative form (RF) DNA of M13 MrΔ-14-35 was linearized by *Bam*HI cleavage within the −14 to −35 deletion, denatured, and then hybridized to single-stranded DNA of M13 MrWT. Heteroduplexes formed contained a short single-stranded region between nucleotides −14 and −35 in the wild-type strand. Deamination of cytosines was performed for 4 hr at 37°C in 3 M sodium bisulfite (12). After dialysis and ethanol precipitation the single-stranded gap was filled in by DNA polymerase I holoenzyme and the DNA was used to transfect *Escherichia coli* JM103. Mutants were identified by sequence determination of single-stranded M13 DNA according to Sanger *et al.* (13).

***In Vitro* Transcription Assays.** The preparation of S100 extracts, the cell-free transcription system, and the analysis of the synthesized RNA have been described (7, 10, 14). The prebinding assay for the identification of transcription complex formation has recently been published (15).

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Abbreviations: rDNA, ribosomal RNA gene; bp, base pair(s); RF, replicative form.

RESULTS

Analysis of Deletion/Substitution Mutants. A previous analysis of deletion mutants of mouse rDNA had indicated that some transcriptional control sequences recognized by RNA polymerase I, transcription factors, or both lie in front of the start site within the region −12 to −39. For a more detailed investigation of the nature of sequences required to achieve accurate transcription *in vitro* we constructed internal deletion/substitution mutants in which the 22 nucleotides between −14 and −35 were replaced by 14-bp foreign DNA, leaving the surrounding rDNA sequences intact (Fig. 1*C*). For functional analysis the wild-type and mutant DNAs were truncated with a restriction enzyme and assayed in the cell-free transcription system. There are unique *Sma* I and *Pvu* II sites in mouse rDNA, located 155 and 292 bp downstream of the start site. Thus, after incubation of the *Sma* I- or *Pvu* II-digested wild-type rDNA in the S100 transcription system, discrete RNA bands 155 or 292 nucleotides long are synthesized (Fig. 1*D*, lanes a and b). An analogous assay of the template activity of mutant pMrΔ-14-35 did not elicit the synthesis of any detectable transcription products (lanes c and d). This finding strongly supports our previous suggestion that an important sequence affecting mouse rRNA synthesis is located within the short region between −14 and −35.

It could be possible that the transcriptional incompetence of mutant pMrΔ-14-35 was due to the 8-bp change in the distance between the initiation site and further upstream regions. To substitute for the missing nucleotides a synthetic decameric oligonucleotide (a *Bam*HI/*Sma* I adaptor) was inserted into the *Bam*HI site of pMrΔ-14-35, yielding the series of deletion/substitution mutants pMrΔ-14BS-$35_{1,\ 2,\ \text{or}\ 3}$, containing one, two, or three adaptor molecules within the deletion (Fig. 1*B*). When these mutants were assayed in the cell-free transcription system each of them proved to be inactive (Fig. 1*D*, lanes e–j). This strongly suggests that the lack of transcriptional activity of these deletion/substitution mutants is due to the removal of the essential rDNA sequences rather than the spacing change.

Construction and Analysis of Point Mutants. In an effort to define the critical nucleotides within the −35 to −14 promoter element, mutants containing single- and multiple-base-pair changes within this region were constructed and analyzed. The strategy employed was a combination of the bisulfite mutagenesis procedure (11) with methods of bacteriophage M13 cloning and sequencing (10). The 324-bp *Sal* I/*Sma* I fragments from pMrWT and mutant pMrΔ-14-35 DNA were cloned in M13 vectors mp8 and mp9, and heteroduplexes were formed and mutagenized as described in *Materials and Methods*. This resulted in mutagenesis of either the coding or the noncoding DNA strand. C-to-T transitions in the coding strand were obtained with M13 mp8, whereas the noncoding strand was mutagenized by using M13 mp9 heteroduplexes. Compilation of sequence data of 15 cloned mutants (Fig. 2*A*) shows that both single and multiple point mutations were produced. All of the possible G·C base pairs within the control region were mutated in various combinations.

To examine the promoter function of the mutants, the double-stranded RF DNAs were cleaved with *Sma* I and used as templates in the cell-free transcription system. A summary of the transcription data giving transcription levels of each mutated DNA relative to that observed with wild-type rDNA is provided in Fig. 2*A*. The data represent mean values of at least five different experiments performed with two DNA concentrations (0.3 and 1 μg/50 μl) in the presence and absence of an internal reference DNA. The reference DNA used was p36.1, a mouse endogenous retroviral clone (16) which contains a 290-bp RNA polymerase III transcription unit (unpublished results). We determined the transcription levels of the mutants relative to a constant amount of RNA polymerase III transcripts, which enabled us to eliminate errors that may be caused by losses during sample work-up or possible inhibitory components in individual DNA preparations.

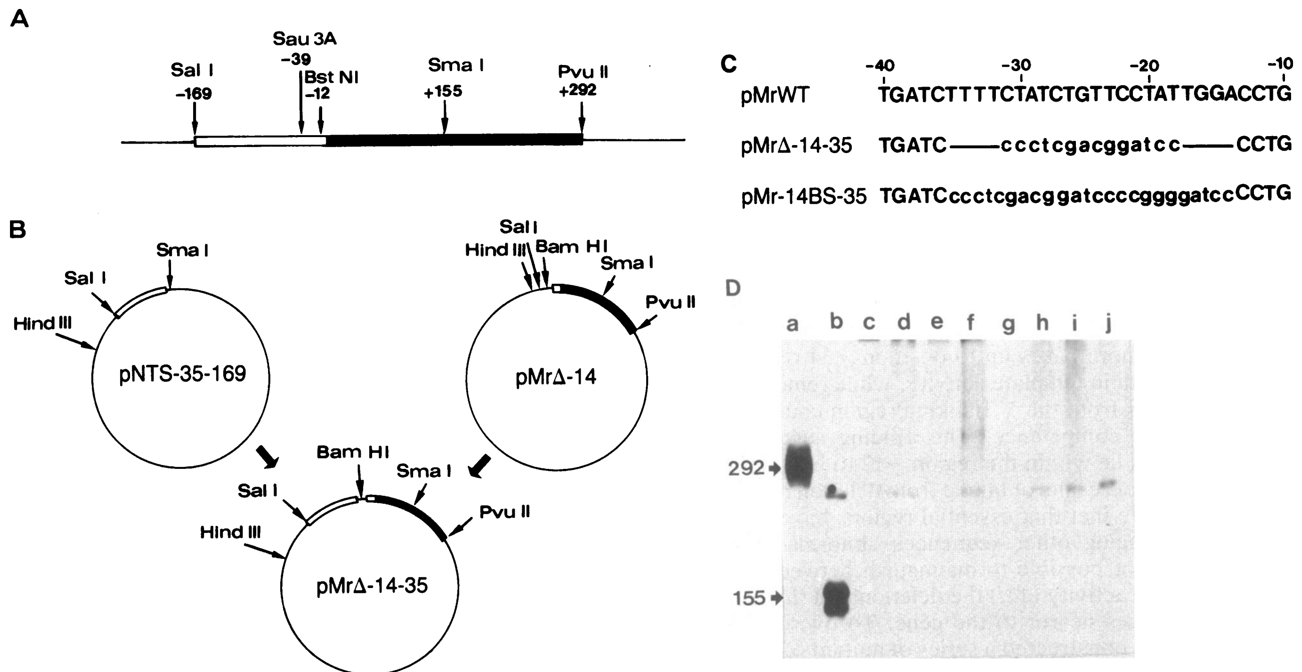

FIG. 1. (*A*) rDNA sequences in pMrSP. Solid bar, external transcribed spacer; empty bar, nontranscribed spacer. Numbers are nucleotides. (*B*) Construction of the mutant pMrΔ-14-35. (*C*) Sequence of the region −40 to −10 of wild-type rDNA as compared to mutant pMrΔ-14-35 and the substitution mutant pMr-14BS-35_1. Lower-case letters indicate the number of adapter molecules inserted. (*D*) Transcription of wild-type and mutant DNAs truncated with *Pvu* II (lanes a, c, e, g, and i) or *Sma* I (lanes b, d, f, h, and j). The templates used (5 μg/ml) were pMrSP (lanes a and b), pMrΔ-14-35 (lanes c and d), pMr-14BS-35 (lanes e and f), pMr-14BS-35_2 (lanes g and h), and pMr-14BS-35_3 (lanes i and j). The weak band below the 292-nucleotide run-off RNA is a nonspecific RNA that originates from vector sequences.

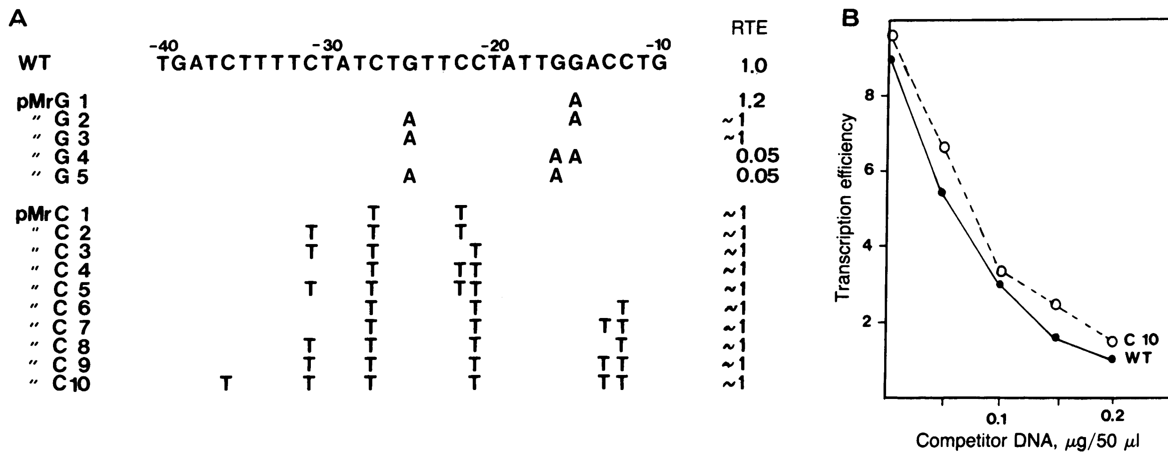

FIG. 2. Sequence analysis and transcriptional activity of rDNA point mutants. (*A*) The positions of base changes found in the mutants are shown below the wild-type rDNA sequence. The relative transcription efficiency (RTE) of the mutants as determined by *in vitro* transcription and densitometric scanning of the autoradiograms was compared to that of wild-type DNA. (*B*) Competition of M13 wild-type and mutant pMrC10 DNA with wild-type pMrSP DNA. M13 WT and pMrC10 DNAs (0.3 μg), truncated with *Sma* I, were assayed in the cell-free transcription system in the presence of increasing amounts of competitor DNA pMrSP/*Pvu* II (0–0.2 μg/50 μl). The total DNA concentration per assay was kept at 0.5 μg/50 μl by adding various amounts of nonspecific pUC9 DNA that had been linearized with *Eco*RI. The amount of the 155-nucleotide transcripts is plotted against the amount of competitor DNA.

Most of the mutants with altered template activity contain more than one mutation in the control region. To correlate the altered transcriptional activity with sequence changes, mutants with common nucleotide alterations were compared to determine the effects of particular point mutations.

The mutants pMrG4 and pMrG5 show a 95% decrease in template activity. They have G-to-A transitions at positions −15/−16 and −16/−25, respectively. Because the single base change at −15 (pMrG1) actually increases template activity, and a mutation at −25 (pMrG3) has no appreciable effect, we attribute the profound reduction in transcription to the single base alteration at G −16. A different result was obtained for the C series. All of the mutants pMrC1–10 produced run-off transcripts with an efficiency comparable to that of wild-type DNA. No mutants were isolated with only a single base substitution. Multiple changes ranging from two to six C-to-T transitions were obtained. Even the most heavily mutated DNA (pMrC10) showed the same template activity as the wild type. Although we cannot exclude the possibility that the effects of multiple base exchanges could be compensatory rather than additive, the similarity of the phenotypes of all the different C mutants strongly suggests that C-to-T transitions at positions −36, −31, −27, −22, −21, and −13 do not affect transcription. We also investigated whether under competitive conditions the C mutants showed transcriptional activity similar to that of the wild type. For this, M13 WT or C10 DNA was truncated with *Sma* I and assayed in the presence of increasing amounts of pMrSP that had been cleaved with *Pvu* II. As shown in Fig. 2*B*, the same competition curves were obtained. The fact that the wild-type DNA pMrSP competes much better than either WT or mutant M13 DNA is due to (*i*) the lower molecular weight of the plasmid pMrSP compared to the M13 clones and (*ii*) the fact that *Pvu* II-cleaved templates are always more efficiently transcribed than rDNA clones truncated with *Sma* I (see also Fig. 3).

Transcriptional Efficiencies of Mutant DNAs Correlate with Their Abilities to Form Transcriptional Complexes. The differences in transcriptional activities of the various mutants could be due to an altered interaction of a specific DNA-binding factor with essential sequences between −14 and −35. To test this possibility we used the so-called prebinding assay, which has been described (15). A short incubation of rDNA with S100 extracts results in stable binding of transcription factor(s) to specific DNA, preventing or reducing transcription of any subsequently added rDNA template. If, however, the first rDNA template lacks sequences necessary for specific binding, the second DNA template will be transcribed efficiently. This assay was used to compare the efficiencies of wild-type and mutant DNAs in forming stable complexes. pMrΔ-14-35 and three point mutants were used, having increased (pMrG1) or strongly inhibited (pMrG4 and pMrG5) template activities. They were recloned from M13 in pUC9, digested with *Eco*RI (allowing a potential 155-nucleotide run-off RNA), and used as prebinding DNA templates.

An equimolar mixture of wild-type DNA pMrSP (14) truncated with either *Pvu* II or *Sma* I yielded both the 297- and

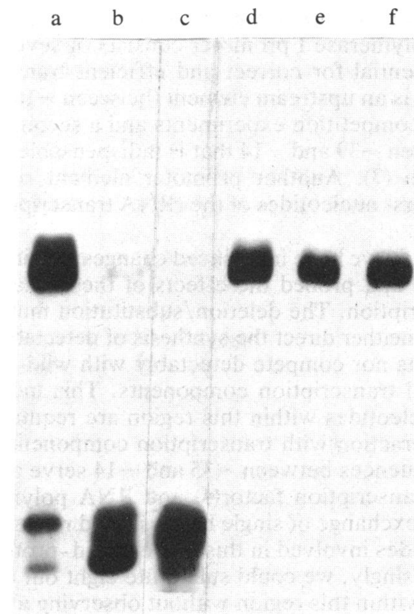

FIG. 3. Competition of wild-type and mutant templates for transcription factors. Lane a, simultaneous incubation of pMrSP/*Sma* I and pMrSP/*Pvu* II. Lanes b–f, assembly of transcription complexes by preincubation of the first DNA (10 μg/ml) for 10 min in the absence of NTPs; the first DNAs were pMrSP/*Sma* I (lane b), pMrG1/*Sma* I (lane c), pMrG4/*Sma* I (lane d), pMrG5/*Sma* I (lane e), and pMrΔ-14-35/*Sma* I (lane f). After the preincubation period, pMrSP/*Pvu* II at 10 μg/ml and NTPs (including [α-^{32}P]GTP) were added and transcription was terminated 60 min later.

		-40 · -30 · -20 · -10 · +1 · +10
Mouse	(10)	TGATCTTTTCTATCTGTTCCTATT**G**GACCTGGAGATAGG**T**ACTGACACGC
Rat	(12)	TCATCTTTGCTATCTGT.CCTATT**G**TACT.GGAGATATA**T**GCTGACACGC
Human	(17)	TTTCGCTCCGAGTCGGCAATTTTG**G**GCCGCCGGGTTATA**T**GCTGACACGC
X. laevis	(18)	CTCCATGCTACGCTTTTTTGGCAT**G**TGCGGGCAGGAAGG**T**AGGGGAAGAC
X. clivii	(19)	CTCTAATCTACGCGTTTTAGGCAT**G**TGCCGACAGGAAGG**T**AGGGAGAGAA
X. borealis	(20)	CGCCATGCTACGCTTTTCGGGTAC**G**TGCGGACAGGAAGG**T**AGGGAGAGGC
D. melanogaster	(21)	AAATACCCGCTTTGAGGACAGCGG**G**TTCAAAAACTACTA**T**AGGTAGGCAG
T. pyriformis	(22)	AAAAAAAAAAAAAGTATCAGGGGG**G**TAAAAATGCATATT**T**AAGAAGGGAA

FIG. 4. Comparison of sequences flanking the rDNA initiation site in several species. *X.*, *Xenopus*; *D.*, *Drosophila*; *T*, *Tetrahymena*. Numbers in parentheses are references.

155-nucleotide RNA (Fig. 3, lane a). However, when pMrSP/*Sma* I DNA was preincubated for 10 min at 30°C, transcription from the second template pMrSP/*Pvu* II was virtually abolished (lane b). This preferential transcription of the first template was also observed for the point mutant pMrG1, which had shown increased template activity (lane c). Actually, this mutant competed approximately 20% more effectively for transcription factor(s) than the wild-type DNA, as revealed by a series of competition experiments (not shown). However, when the mutant DNAs pMrG4, pMrG5, or pMr-14-35 were used in the prebinding assay, transcription of the subsequently added wild-type DNA pMrSP/*Pvu* II was not affected (lanes d–f). This indicates that transcription factors present in the S100 extracts bind to and remain associated with wild-type and pMrG1 DNA, but do not bind, or only weakly interact with, the transcriptionally inactive mutant DNAs.

DISCUSSION

Previous analysis of rDNA deletion mutants has shown that the RNA polymerase I promoter consists of several regions that are essential for correct and efficient transcription *in vitro*. There is an upstream element (between −169 and −39) defined by competition experiments and a second region located between −39 and −14 that is indispensable for *in vitro* transcription (7). Another promoter element seems to lie within the first nucleotides of the rRNA transcription unit (9, 17).

In this study we have introduced changes within the region −35 to −14 and probed the effects of these changes by *in vitro* transcription. The deletion/substitution mutant pMrΔ-14-35 could neither direct the synthesis of detectable amounts of transcripts nor compete detectably with wild-type rDNA for essential transcription components. This indicates that specific nucleotides within this region are required for productive interaction with transcription component(s).

If the sequences between −35 and −14 serve as a contact point for transcription factor(s) and RNA polymerase I, a systematic exchange of single bases should reveal the essential nucleotides involved in this nucleic acid–protein interaction. Surprisingly, we could substitute eight out of nine G·C base pairs within this region without observing a deleterious effect on transcription. The phenotype of mutant pMrC10, which has six C residues converted to T residues, is identical to that of wild-type DNA in respect to its transcription efficiency at different ionic conditions and DNA concentrations as well as its ability to compete for limiting transcription factors present in the mouse cell extracts.

In contrast, a single base change at G −16 proved to be a strong down mutation. A 95% reduction in transcription and a similar decrease in the ability to compete for transcription components was observed. This finding suggests that this G plays an important role in the interaction of a transcription factor with the rDNA promoter. In this context it is interesting that this G at position −16 seems to be highly conserved in evolution. Fig. 4 shows a comparison of rDNA sequences flanking the 45S pre-rRNA initiation site of several eukaryotes. Since there is no significant sequence conservation, it seems unlikely that a particular consensus promoter sequence can be derived from comparative studies using distantly related species. This lack of homology is in accord with the observation that at least one of the factors required for rDNA transcription by RNA polymerase I is species specific (23). In spite of this obvious sequence divergence, a T at position −1 and the G at −16 is found in all known rDNA sequences of higher organisms. This observation, together with our results about the functional importance of G −16, strongly suggests that these two nucleotides may play an essential role in the interaction of transcription components with the RNA polymerase I promoter. We have no experimental data yet about the role of T −1 for RNA polymerase I promoter function; oligonucleotide-primed mutants in which the T at position −1 is changed might provide such data. Furthermore, the studies described in this paper did not address the question, which of the nucleotides between −34 and −39 is responsible for the reduction of transcription efficiency to 1/10th seen after deletion of sequences between −34 and −39. Further progress towards understanding the mechanism and regulation of rRNA synthesis will be greatly facilitated by a more complete mutational analysis of rDNA promoter sequences as well as the purification and functional characterization of the proteins that interact with these sequences.

We thank the Deutsche Forschungsgemeinschaft and the Fond der Chemischen Industrie for financial support of this work.

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