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Domains of the Rat rDNA Promoter Must Be Aligned Stereospecifically

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Efficient transcription from the rat rDNA promoter results from an undefined interaction between the core (CPE) and upstream (UPE) promoter elements or the protein complexes which form on them. These interactions were demonstrated by the behavior of promoters that contained either linker-scanning or deletion mutations of the UPE in combination with point mutations of the CPE (bidomain mutants). In vivo transcription experiments using point mutations within the CPE (G→A mutation at either −16 or −7) demonstrated that the CPE may in fact consist of two domains. Whereas both of these mutants were rescued by the addition of UBF to in vitro transcription reactions, the CPE mutant −7A/G was inactive in vivo. Experiments with these bidomain mutants demonstrated that the UPE was required for the rescue of the CPE mutants. We also examined the hypothesis that this interaction might require a stereospecific alignment of the promoter elements. Our results indicate that the promoter consists of several domains with differing responses to mutations that alter the distance between, or within, the promoter elements. For example, the insertion or deletion of half-multiples of the helical repeat distance between −167 and −147 had no significant effect on transcription. On the other hand, some sites were sensitive to deletions of any size but not to insertions of up to 20 bp. The analyses of two sites yielded results suggesting that they lay between domains of the promoter that must be on the same side of the DNA helix for promoter activity. The first of these sites mapped between −106 and −95. The second site that demonstrated a cyclical response to distance-altering mutations lies between −60 and −55, i.e., between the 3′ border of the UPE and the 5′ boundary of the CPE. Insertions or deletions of half-multiples of the helical repeat within these sites inactivated the promoter, whereas the insertion or deletion of 10 or 11 bp was permitted. These observations indicate that there is a required stereospecific alignment between the UPE and the CPE and possibly within the UPE.

Basal levels of transcription of the vertebrate ribosomal genes in vivo requires at least two nucleoprotein complexes. One of these forms on the core promoter element (CPE) and is sufficient to direct initiation in vitro, and it is required for transcription in vivo. The second complex forms on the upstream promoter element (UPE) and is required for transcription in vivo and for efficient transcription in vitro. It is unclear whether a third element, the promoter-proximal terminator (T_0), located at −167 in the rat promoter, is a component of the promoter (2, 13, 17, 18, 26, 27). Besides RNA polymerase I, and associated proteins such as TFIC (24), at least two transcription factors are required for efficient transcription in vitro. SL-1, or PC-D, defined in part by its ability to reprogram heterologous systems (29, 37), appears to interact with both the UPE and the CPE and is required for transcription (3, 37). A second factor, UBF, is required for efficient transcription (3, 4, 23, 37). The human and rat forms of UBF footprint over the UPEs of their respective promoters, and the UPE is required for the full effect of UBF on transcription (4, 37).

Both in vitro and in vivo studies clearly suggest an interaction between the two domains of the vertebrate rDNA promoters. In vitro studies indicate that the UPE is required for the formation of a stable preinitiation complex (5) and for increasing the resistance of the initiation process to increasing concentrations of KCl (40). Furthermore, an intact UPE is required for the full effect of UBF on transcription (4, 37, 43, 44). In vivo studies using 5′ deletion mutants have demonstrated that the UPE is required for expression in vivo (19, 36). This has been confirmed by studies using linker-scanning mutants of the human (15, 16), *Xenopus* (32, 41), and rat rDNA promoters (44). In this report, we demonstrate this interaction with constructs combining UPE and CPE mutants. Just how these domains interact is not clear.

To address this question, we undertook a systematic study of the effects of distance-altering mutations throughout the rat rDNA promoter. Previous studies of the human rDNA promoter suggested that studies of the spacing of the UPE and CPE relative to one another might be revealing (15, 16, 36). However, the placement of the distance-altering mutations within the human promoter and the sizes of the alterations created (4 bp or greater than 28 bp) did not allow for definitive conclusions. We report here evidence suggesting that (i) the 3′ boundary of the UPE lies at about −54 (+1 being the transcription initiation site of the rat rDNA promoter), in agreement with the footprint of UBF over the rat promoter (38); (ii) there may be a specific alignment required within the UPE; and (iii) there is a required stereospecific orientation of the UPE and the CPE; i.e., elements must be on the same side of the helix.

Our results suggest that rDNA transcription results from the interaction of paired promoter elements and reflects the requirement that the nucleoprotein complexes that form on the UPE and CPE bind to the same face of the DNA helix. This observation is similar to those for model systems such as the λ repressor (20), the *araBAD* regulatory region (10), the adenovirus promoter (42), and the simian virus 40 early promoter (39). These results contrast with the characterization of the interactions of the *cis*-acting elements of the

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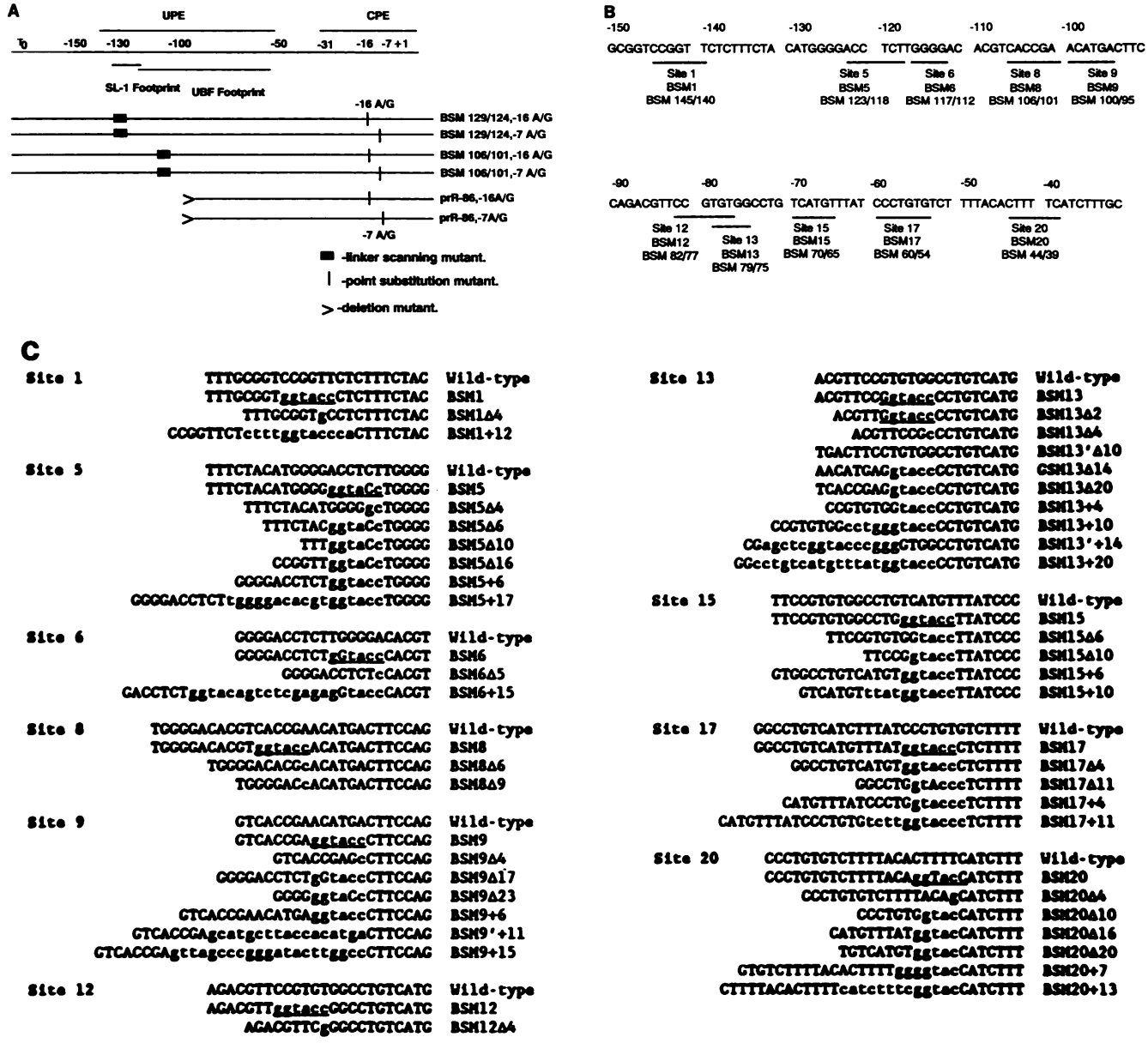

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Site 1	TTTGCCGTCCCGTTCTCTTTCTAC	Wild-type
	TTTGCCGTggtaccCTCTTTCTAC	BSM1
	TTTGCGGTgCCTCTTTCTAC	BSM1Δ4
	CCGGTTCTctttggtaccceaCTTTCTAC	BSM1+12
Site 5	TTTCTACATGCGGACCTCTTGGGG	Wild-type
	TTTCTACATGGGGggtaCcTGGGG	BSM5
	TTTCTACATGCGGgcTGGGG	BSM5Δ4
	TTTCTACggtaCcTGGGG	BSM5Δ6
	TTTggtaCcTGGGG	BSM5Δ10
	CCGGTTggtaCcTGGGG	BSM5Δ16
	GGGGACCTCTggtaccTGGGG	BSM5+6
	GGGGACCTCTtggggacacgtggtaccTGGGG	BSM5+17
Site 6	GGGGACCTCTTGGGGACACGT	Wild-type
	GGGGACCTCTgGtaccCACGT	BSM6
	GGGGACCTCTcCACGT	BSM6Δ5
	GACCTCTggtacagtctcgagagGtaccCACGT	BSM6+15
Site 8	TGGGGACACGTCACCGAACATGACTTCCAG	Wild-type
	TGGGGACACGTggtaccACATGACTTCCAG	BSM8
	TGGGGACACGcACATGACTTCCAG	BSM8Δ6
	TGGGGACcACATGACTTCCAG	BSM8Δ9
Site 9	GTCACCGAACATGACTTCCAG	Wild-type
	GTCACCGAggtaccCTTCCAG	BSM9
	GTCACCGAGcCTTCCAG	BSM9Δ4
	GGGGACCTCTgGtaccCTTCCAG	BSM9Δ17
	GGGGggtaCcCTTCCAG	BSM9Δ23
	GTCACCGAACATGAggtaccCTTCCAG	BSM9+6
	GTCACCGAgcatgcttaccacatgaCTTCCAG	BSM9'+11
	GTCACCGAgttagcccgggatacttggccCTTCCAG	BSM9+15
Site 12	AGACGTTCCGTGTGGCCTGTCATG	Wild-type
	AGACGTTggtaccGGCCTGTCATG	BSM12
	AGACGTTCgGGCCTGTCATG	BSM12Δ4
Site 13	ACGTTCCGTGTGGCCTGTCATG	Wild-type
	ACGTTCCGgtaccCCTGTCATG	BSM13
	ACGTTGgtaccCCTGTCATG	BSM13Δ2
	ACGTTCCGcCCTGTCATG	BSM13Δ4
	TGACTTCCTGTGGCCTGTCATG	BSM13'Δ10
	AACATGACgtaccCCTGTCATG	GSM13Δ14
	TCACCGACgtaccCCTGTCATG	BSM13Δ20
	CCGTGTGGtaccCCTGTCATG	BSM13+4
	CCGTGTGGcctgggtaccCCTGTCATG	BSM13+10
	CGagctcggtacccgggGTGGCCTGTCATG	BSM13'+14
	GGcctgtcatgtttatggtaccCCTGTCATG	BSM13+20
Site 15	TTCCGTGTGGCCTGTCATGTTTATCCC	Wild-type
	TTCCGTGTGGCCTGggtaccTTATCCC	BSM15
	TTCCGTGTGCtaccTTATCCC	BSM15Δ6
	TTCCGgtaccTTATCCC	BSM15Δ10
	GTGGCCTGTCATGTggtaccTTATCCC	BSM15+6
	GTCATGTttatggtaccTTATCCC	BSM15+10
Site 17	GGCCTGTCATCTTTATCCCTGTGTCTTTT	Wild-type
	GGCCTGTCATGTTTATggtaccCTCTTTT	BSM17
	GGCCTGTCATGTggtaccCTCTTTT	BSM17Δ4
	GGCCTGgtAcccTCTTTT	BSM17Δ11
	CATGTTTATCCCTGgtacccTCTTTT	BSM17+4
	CATGTTTATCCCTGTGtcttggtacccTCTTTT	BSM17+11
Site 20	CCCTGTGTCTTTTACACTTTTCATCTTT	Wild-type
	CCCTGTGTCTTTTACAggTacCATCTTT	BSM20
	CCCTGTGTCTTTTACAgCATCTTT	BSM20Δ4
	CCCTGTGgtacCATCTTT	BSM20Δ10
	CATGTTTATggtacCATCTTT	BSM20Δ16
	TGTCATGTggtacCATCTTT	BSM20Δ20
	GTGTCTTTTACACTTTTggggtacCATCTTT	BSM20+7
	CTTTTACACTTTTcatctttcggtacCATCTTT	BSM20+13

FIG. 1. Schematic depiction of the mutants used. (A) Schematic representation of the bidomain mutants. Except where indicated, the constructs depicted are identical to the rat wild-type promoter except that the *Xho*I site (+634) of the wild-type promoter has been converted into an *Eco*RI site. T_0, the promoter-proximal terminator, includes the *Sal*I site at −167. (B) Linear presentation of the sites of the distance-altering mutants used. The 5′ end of the UPE lies circa −147, and the UPE extends to approximately −50. (C) Sequences of the distance-altering mutants constructed at each site. Also presented are the sequence of the wild-type DNA and the parental linker-scanning mutant. The *Kpn*I site of each mutant is underlined; all insertions and substitutions are in lowercase.

chicken skeletal muscle α-actin gene promoter (8). In the latter report, altering the stereospecific alignment of individual promoter elements by half-helical distances, i.e., mutations that would move them to opposite sides of the helix, stimulated transcription. Such a mutation between the UPE and the CPE of the rat promoter would inactivate the promoter.

MATERIALS AND METHODS

Templates. The bidomain mutants (Fig. 1) were constructed by using polymerase chain reaction oligonucleotide-directed site mutagenesis (1) to introduce the desired point mutation into the indicated parental constructs. Primer pair 1, 5′-CTTATTA(−16)TACCTGGAGATATATGCTG-3′ (primer 1) and 5′-GACAGATAGCAAAGATG-3′ (primer 2), was used to introduce a point mutation, G→A (−16), into the linker-scanning mutants BSM 124/126 and BSM 106/101 and the deletion mutant prR-86. Primer pair 2, CTTATTGT ACCTGGAA(−7)ATATATGCTG (primer 3) and primer 2, was used to introduce a point mutation, G→A (−7), into the same three parental mutants.

The distance-altering mutants used in these studies (Fig. 1) were created from a *Kpn*I site-based linker-scanning mutant

series of the rat rDNA promoter (44). To create small deletions, the constructs were opened at the *Kpn*I site, treated with T4 DNA polymerase in the presence of all four deoxynucleoside triphosphates, and recircularized by blunt-end ligation. The resulting clones were then sequenced and named by the linker-scanning mutant that was the progenitor. To create larger deletions and insertions, the fragment extending from a *Bam*HI site (at −286) to the desired *Kpn*I site was ligated to a complementary fragment extending from its *Kpn*I site through the vector to the *Bam*HI site. In some cases, larger insertions were created by ligating synthetic adapters into the requisite *Kpn*I site or created by oligonucleotide-directed site mutagenesis via polymerase chain reaction. All mutations were confirmed by dideoxy sequencing (35). The pseudo-wild-type promoter used in the transfection experiments was generated by site-directed mutagenesis using the polymerase chain reaction and contains a 9-bp insertion at +20. Routine DNA manipulations were carried out as described previously (34).

In vitro transcription. In vitro transcription was carried out essentially as described previously (33, 37), using nuclear extracts of Novikoff hepatoma ascites cells (14). The mutant templates were truncated at a synthetic *Eco*RI site to yield a 638-nucleotide (nt) transcript. The wild-type template was truncated at a different synthetic *Eco*RI site to yield a 570-nt transcript. The transcription products were resolved by denaturing polyacrylamide gel electrophoresis, analyzed on an AMBIS radioanalytic analyzer, and then subjected to autoradiography.

For in vitro assays demonstrating the cooperativity of the UPE and the CPE, the bidomain mutants were assayed by using a UBF-depleted fraction (DE-175) and UBF purified through the carboxymethyl-Sephadex column chromatography step, as described previously (33).

Transfection. Chinese hamster ovary cells (CHO) were used for the analysis of the activities of the mutant promoter constructs in vivo. The CHO cells were maintained and cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. One day before transfection, 5×10^5 cells were plated on 60-mm Falcon tissue culture plates. The plasmid DNAs used for transfection were purified through two rounds of CsCl ultracentrifugation. For each transfection, 2 μg of plasmid DNA carrying the test promoter and 2 μg of plasmid DNA carrying a pseudo-wild-type promoter were used. Transfection was performed by using DEAE-dextran essentially as described previously (1). The pseudo-wild-type gene is the same as the wild-type gene except for an insertion of 9 bp at +20 and therefore served as an internal control for the efficiency of transfection and for the reverse transcriptase assays.

At 20 to 24 h after transfection, total RNA was isolated from transfected cells essentially as described previously (7). Twenty micrograms of the whole cell RNA isolated from each plate and 5×10^5 cpm of a ^{32}P-labeled primer (5′-GCTGGACAAGCAAACAGCC) specific for the 5′ terminus of the rat 45S rRNA (43) were used to perform primer extension assays (45).

The elongation products were resolved by electrophoresis on sequencing gels and detected by autoradiography. Gels were quantitated with an AMBIS radioanalytic analyzer. In some instances, there are visible autoradiographic images of the transcripts from the mutant template, yet we indicate that the mutant template was essentially inactive because the AMBIS was unable to detect a significant number of disintegrations above background.

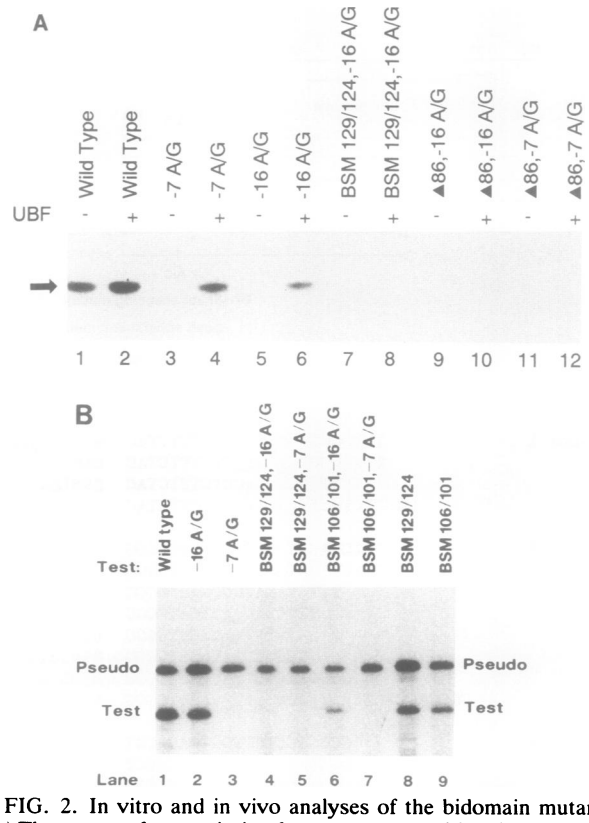

FIG. 2. In vitro and in vivo analyses of the bidomain mutants. (A) The rescue of transcription from promoters with point mutations in the CPE requires an intact UPE. The indicated promoter constructs (see Fig. 9) were transcribed with a UBF-depleted extract (lanes 1, 3, 5, 7, 9, and 11) or with the same extract supplemented with carboxymethyl-Sephadex-purified rat UBF (33). Each template was truncated at the same *Eco*RI site to yield a 638-nt transcript. (B) In vivo expression assays demonstrate different domains of the CPE as well as an interaction between the UPE and the CPE. CHO cells were cotransfected with the mutant promoter constructs indicated (see Fig. 9) (Test) and a pseudo-wild-type promoter (Pseudo). At 20 to 24 h after transfection, total cellular RNA was isolated and subjected to a primer elongation assay, and the resultant end-labeled DNAs were analyzed by electrophoresis on a sequencing gel and autoradiography. The elongation product of the transcript from the pseudo-wild-type promoter (Pseudo) is 9 bp longer than that from the mutant promoters (Test).

RESULTS

Bidomain mutants demonstrate the interactions between the UPE and the CPE. We have reported that point mutants of the CPE, either the conversion of the guanine at −7 to an adenine, prR-7A/G, or the conversion of the guanine at −16 to an adenine, prR-16A/G, weakened the CPE, such that transcription from those constructs was undetectable in the absence of UBF (33) (Fig. 2A, lanes 3 and 5). The addition of UBF to the transcription assays restored promoter activity in vitro (Fig. 2A, lanes 4 and 6). Although the effects of UBF on these promoter rescue assays are apparent, the mechanism by which UBF is acting is not. Is UBF acting directly on the CPE, or is it acting through the interaction between the UPE and the CPE? One way to address this question is to assess the activity of mutants that contain mutations in both the UPE and the CPE. Since deletion or linker-scanning mutants of the UPE are active either in vitro or in vivo, placing a point mutation of the CPE into such an environ-

ment might allow one to determine whether the effect of UBF is directly on the CPE or whether it is acting in conjunction with the UPE. We have shown previously that transcription from either the deletion mutant prR-86 or the constructs BSM 129/126 (BSM 4) and BSM 106/101 (BSM 8) was not stimulated when UBF was added to transcription reactions using UBF-depeleted extract (33). Therefore, we constructed a series of bidomain mutants (Fig. 1), i.e., mutants that combined a deletion or linker-scanning mutation of the UPE with a point substitution mutation of the CPE, and assayed them both in vivo and in vitro.

The bidomain mutants BSM 129/126,-16A/G, prR-86,-7A/G, and prR-86,-16A/G were inactive in vitro in the absence of UBF and were not rescued by the addition of UBF to the assay system (Fig. 2A, lanes 7 to 12). These results suggest that the rescue of transcription from promoters with weakened core promoter elements does indeed require a functional UPE.

The behavior of these mutant promoter constructs was then assayed in vivo (Fig. 2B). The promoter constructs with mutations in only one domain, such as prR-16A/G, BSM 129/124, and BSM 106/101, were 80, 40, and 50%, respectively, as active as the cotransfected wild-type promoter (Fig. 2B, lanes 2, 8, and 9). However, prR-7A/G was inactive (Fig. 2B, lane 3). The bidomain mutants BSM 129/126,-16A/G, BSM 129/124,-7A/G, and BSM 106/101,-7A/G were inactive in vivo (Fig. 2B, lane 4, 5, and 7). On the other hand, the bidomain mutant BSM 106/101,-16A/G was 70% as active as the wild-type promoter. Deletion mutants containing less than 147 bp of DNA upstream of +1, e.g., prR-86, were not active in vivo (data not shown), and therefore we did not assess prR-86,-16A/G and prR-86,-7A/G in this assay.

Distance-altering mutations demonstrate a required stereospecific alignment of the promoter elements. To determine the sensitivity of the rat rDNA promoter to mutations that would alter alignments around the helix, 4-bp deletions (approximately one half-helical turn) were introduced into four different sites across the rat rDNA promoter. The nomenclature of the distance-altering mutants refers to the parental linker-scanning mutant in terms of its distance from the transcription initiation site, with lower numerical values designating mutants further from +1. For example, BSM 1Δ4 is a 4-bp deletion at site 1 based upon the linker-scanning mutant BSM 1 or BSM 145/140 (see Fig. 1B). Two of the resulting promoter constructs, BSM 1Δ4 and BSM 5Δ4, were functional (Fig. 3, lanes 1 and 3). However, two other deletions, BSM 9Δ4 and BSM 12Δ4, were inactive (Fig. 3, lanes 6 and 7) despite the fact that the parental constructs were fully active (44). We did not observe primer elongation products other than those predicted from initiation at +1.

These results suggested that there were going to be domains of the rDNA promoter with different degrees of sensitivity to distance-altering mutants. To investigate this pattern in greater detail, the series of distance-altering mutants depicted in Fig. 1 was created. The template capacity of each construct was assayed by cotransfecting CHO cells with the mutant construct and a pseudo-wild-type reporter.

When the distance between T_0 (−167) and the 5′ end of the UPE was altered by either deleting 4 bp (BSM 1Δ4) or inserting 12 bp (BSM 1+12) at site 1, there was no significant effect on transcription (Fig. 3, lanes 1 and 2). This result suggests that if T_0 plays a role in transcription, there is not a strict requirement for the distance between T_0 and the remainder of the promoter.

Relatively small deletions between −122 and −112, BSM 5Δ4, BSM 6Δ5, and BSM 5Δ6 (Fig. 3, lanes 3 and 4; Fig. 4, lane 2), reduced promoter activity no more than 60%. On the other hand, the deletion of 4 bp between −100 and −95 (BSM 9Δ4) and between −82 and −77 (BSM 12Δ4) inactivated the promoter (Fig. 3, lanes 6 and 7).

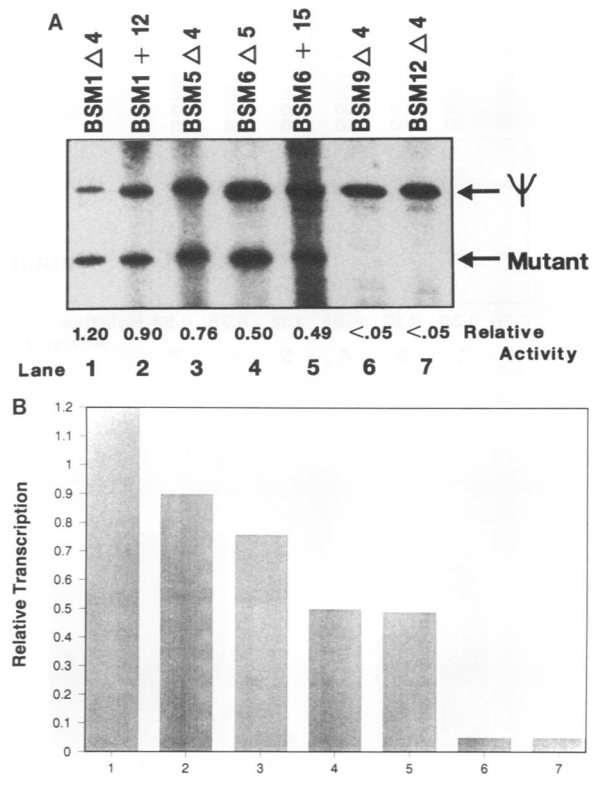

FIG. 3. Analysis showing that some regions of the promoter are relatively resistant to large-scale insertions or small-scale deletions whereas others are sensitive to small scale deletions. Details of the experiment are the same as for Fig. 2B. Ψ, primer elongation product of the pseudo-wild-type promoter; Mutant, the primer elongation product of the distance-altering mutant. The activity of each mutant promoter relative to that of the pseudo-wild-type promoter is presented both below the autoradiograph and in panel B.

In contrast to the effects of small-scale deletions at site 5, larger deletions at the same site, BSM 5Δ10 and BSM 5Δ16, extending further upstream, reduced template capacity by 90 to 95% (Fig. 4, lanes 3 and 4, respectively). However, neither a small insertion (BSM 5+6) nor a larger one (BSM 5+17) at the same site reduced promoter activity by more than 40% (Fig. 4, lanes 6 and 7). The insertion of a random 15-mer at −114 (BSM 6+15) resulted in a similar effect on transcription (Fig. 3, lane 5). These results suggested that the effects of the larger deletions at these sites were probably due not to alterations in critical distances but to the deletion (partial or complete) of *cis*-acting components.

A detailed analysis of the domain between −106 and −95 demonstrated an interesting pattern. The parental mutants for this series, BSM 106/101 (BSM 8) and BSM 100/95 (BSM 9), were transcribed at 50 and 160%, respectively, of the level of the wild-type construct. Approximately half-helical deletions at sites 8 and 9, BSM 8Δ6 and BSM 9Δ4, and insertions, BSM 9+6, were found to inactivate the promoter (Fig. 5, lanes 2, 8, and 5, respectively). On the other hand, approximately full turn deletions at sites 8 and 9, BSM 8Δ9, and insertions, BSM 9+11, were permitted (Fig. 5A, lanes 1 and 6). Larger deletions of 17 bp (BSM 9Δ17) and 23 bp

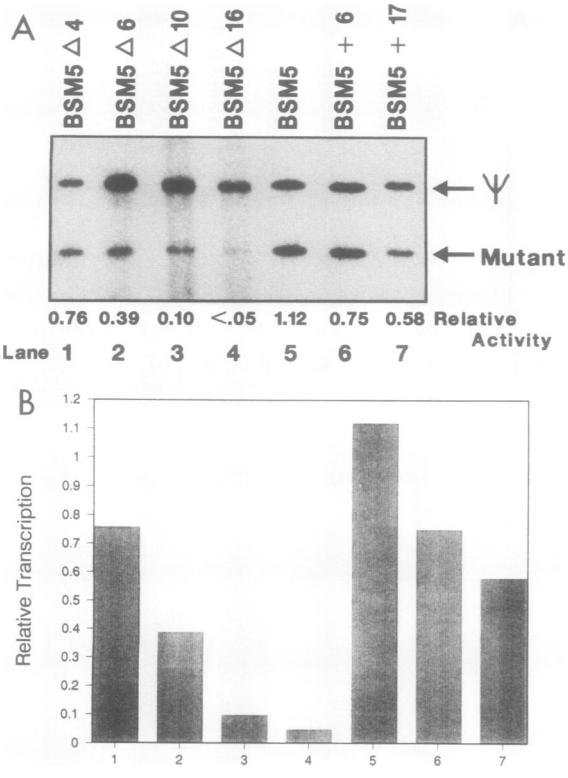

FIG. 4. Evidence that site 5 is sensitive to the effects of larger deletion mutations but relatively insensitive to insertions and smaller deletions. For experimental details, see the legends to Fig. 2 and 3 as well as Materials and Methods.

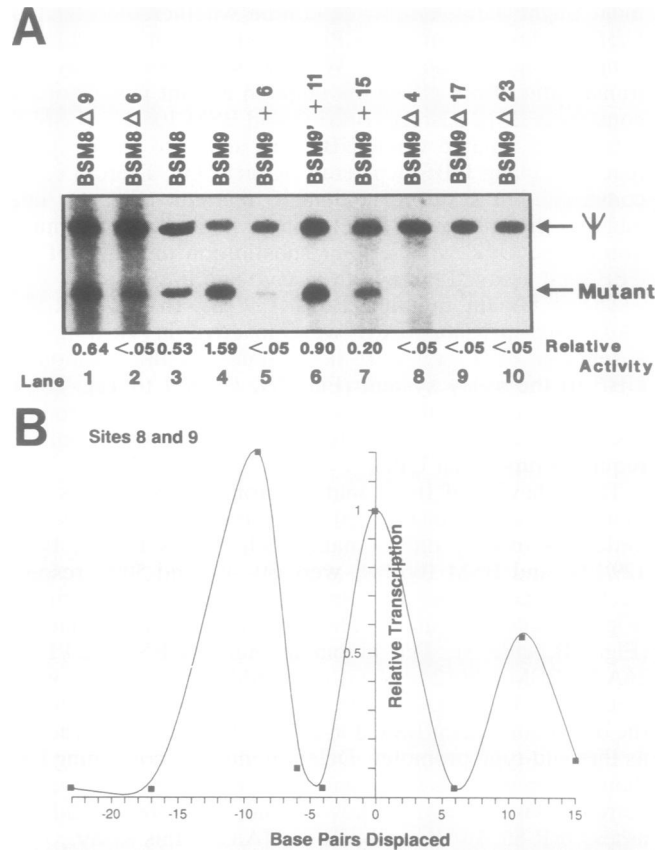

FIG. 5. Evidence that sites 8 and 9 are sensitive to mutations of approximately a half-helical turn but not to mutations of the full helical repeat distance. For experimental details, see the legends to Fig. 2 and 3 as well as Materials and Methods. (B) Graphical analysis of the effects of the distance-altering mutants at sites 8 and 9 on transcription. For the sake of comparison, the activities of BSM 8 and BSM 9, the parental constructs, relative to that of the wild-type promoter were set to 1. Then, the observed levels of transcription of the derivative constructs relative to that of the wild-type construct in each lane were plotted against the *y* axis. The *x* axis indicates the number of base pairs deleted or inserted.

(BSM 9Δ23) were inactive (Fig. 5A, lanes 9 and 10, respectively). While the insertion of 15 bp (BSM 9+15) did not inactivate the promoter, that construct was transcribed at only 20% of the wild-type level (Fig. 5A, lane 7). These results are plotted in Fig. 5B. The cyclical response of promoter activity in relationship to mutations that alter the distance through this region by one-half and full helical turns is apparent.

The analysis of site 13, the domain circa −79 to −75, demonstrated that that region was sensitive to deletions (Fig. 6A, lanes 1 to 5) but not to insertions. The deletion of only 2 nt reduced the template capacity by approximately 40%, and the deletion of 4 bp inactivated the promoter. In contrast, up to 20 bp could be inserted at site 13 and the template capacity was still 60% of the wild-type level (Fig. 6A, lanes 7 to 10).

Analysis of the effects of distance-altering mutations at sites 15 and 17, between −70 and −54, demonstrated a pattern suggesting that this region lies between two domains with a required stereospecific alignment. The parental constructs for these mutants, BSM 70/65 (BSM 15) and BSM 60/54 (BSM 17), were transcribed 160 and 100% as well as the wild-type promoter (Fig. 7A, lane 3; Fig. 8B, column 3). The deletion or insertion of either 6 bp at site 15 (Fig. 7A, lanes 2 and 4) or 4 bp at site 17 (Fig. 7B, lanes 2 and 4) reduced promoter activity more than 90%. However, deletions or insertions of 10 or 11 bp did not significantly alter promoter activity (lanes 1 and 5 in Fig. 7A and B). The quantitative depiction of the response of promoter activity to the effects of distance-altering mutations at these sites is depicted graphically in Fig. 7C.

Site 20, between −44 and −39, did not demonstrate a definitive stereospecific effect (Fig. 9). Instead, this domain was sensitive to deletions (Fig. 8A, lanes 1 to 4) and to a small insertion (+6) (Fig. 8, lane 6) but not to a slightly larger insertion (+13).

The observation of the effects of distance-altering mutants on promoter activity was extended by in vitro transcription experiments (Fig. 9). In these experiments, the test templates were cotranscribed in vitro with a control wild-type template. The test templates were truncated so that they would yield a transcript of 638 nt, and the wild-type template was truncated to result in the synthesis of a 570-nt transcript. In these experiments, we would not expect to see all-or-none effects of distance-altering mutations, as the CPE of the rat rDNA promoter is sufficient to support transcription (5). As we observed in our in vivo assays, BSM 1Δ4 and BSM 5Δ4 were as active as the wild-type promoter (Fig. 9A, lanes 1 and 2), while BSM 13Δ4 was transcribed at 25% of the wild-type level (Fig. 9, lane 3). Both BSM 13+10 and BSM 13Δ10 behaved similarly in the in vitro assay and the in vivo assays (Fig. 9A, lanes 4 and 5). BSM 20Δ4 was also a down

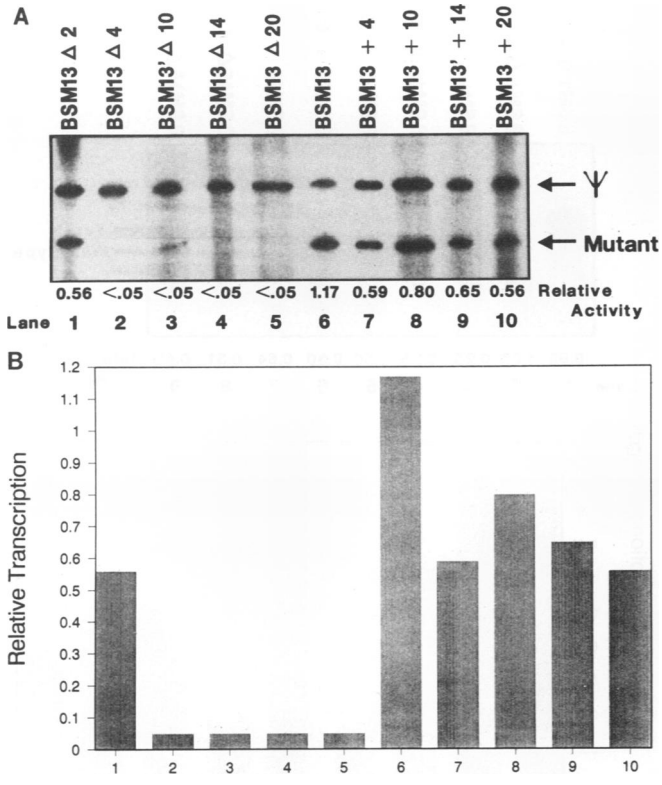

FIG. 6. Inhibition by deletions but not by insertions of in vivo transcription of distance-altering mutants at site 13. For experimental details, see the legends to Fig. 2 and 3 as well as Materials and Methods.

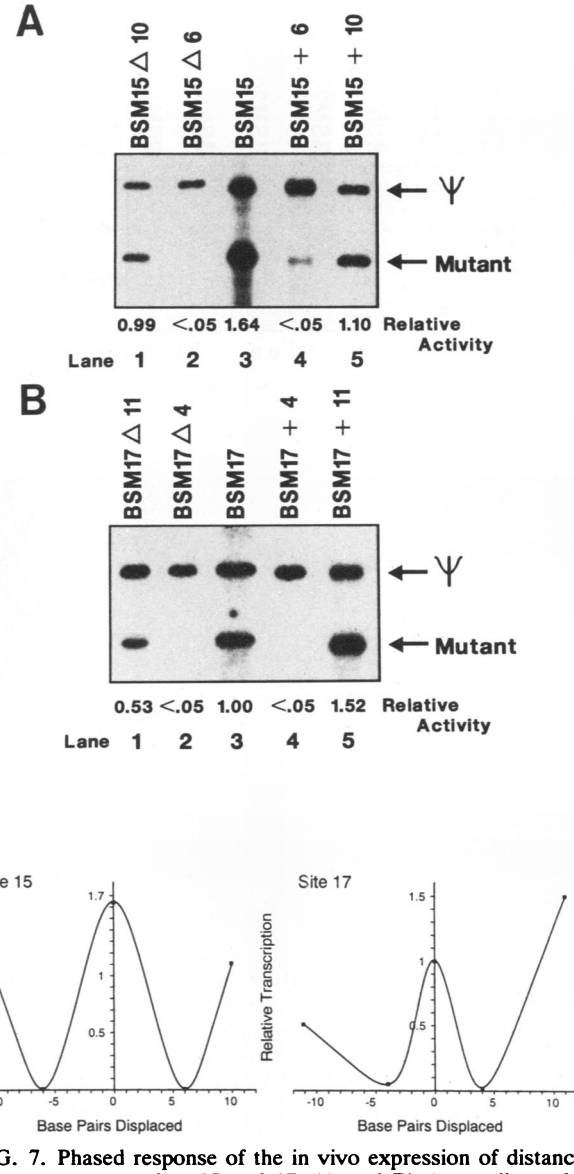

FIG. 7. Phased response of the in vivo expression of distance-altering mutants at sites 15 and 17. (A and B) Autoradiographic analysis of the in vivo expression of distance-altering mutants at sites 15 and 17. For experimental details, see the legends to Fig. 2 and 3 as well as Materials and Methods. (C) Graphical analysis of the relative levels of transcription of the mutants at sites 15 and 17. The x axis indicates the number of nucleotides inserted or deleted, and the y axis indicates the transcription of the mutant promoter relative to the transcription of the cotransfected wild-type promoter.

mutant in vitro (data not shown). The distance-altering mutants at sites 15 and 17 behaved slightly differently in vitro than they behaved in vivo. These results, for site 15, are presented in Fig. 9, lanes 6 to 9. Both full- and half-helical insertions or deletions were down mutations. However, the promoter constructs with full-helical-turn deletions or insertions had higher residual levels of activity than did those with half-turn deletions or insertions (Fig. 9A; compare the levels of transcription in lanes 6 and 8 with those in lanes 7 and 9).

DISCUSSION

We have examined the interactions between the *cis*-acting elements of the rat rDNA promoter by using two different classes of promoter mutants. One class, the bidomain mutants, demonstrated the essential nature of the interactions of the UPE and CPE. Studies of the distance-altering mutants demonstrated a required stereospecific alignment between the promoter elements. The in vivo experiments using the point mutants in the CPE appear to demonstrate a functional difference between the guanines at −16 and −7. In our in vitro experiments (33; this report), prR-16A/G and prR-7A/G behaved similarly, both in the UBF rescue assays and when transcribed by whole cell extracts.

On the other hand, in the in vivo assays, the transition from G→A at −16 in the CPE weakened the promoter, while the same transition at −7 was lethal to promoter activity. This is the first evidence we have observed that qualitatively discriminates between the guanines at positions −16 and −7 of the rat rDNA promoter. Guanines are found in essentially the same two positions in virtually all of the vertebrate promoters transcribed by RNA polymerase I (18). In vitro studies of the mouse rDNA promoter indicated that mutating either of these guanines reduced transcription in vitro to 10% or less of the level supported by the wild-type promoter (9). Although neither the in vitro nor the in vivo studies have demonstrated a quantitative effect for the G at −16 of the rat rDNA promoter, the in vivo studies do suggest that the two guanines do not participate in the same way in transcription. This may be explained by observations of the behavior of the mouse rDNA transcription factor TIF-1B or SL-1. The guanine at −16 has been reported to be essential for the

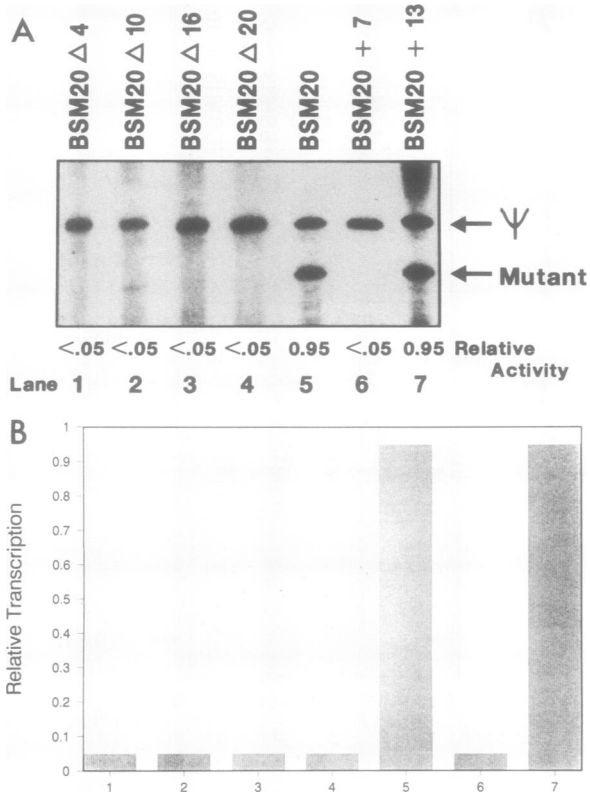

FIG. 8. Analysis of the effects of distance-altering mutants at site 20 on transcription of the rat rDNA promoter in vivo. For experimental details, see the legends to Fig. 2 and 3 as well as Materials and Methods.

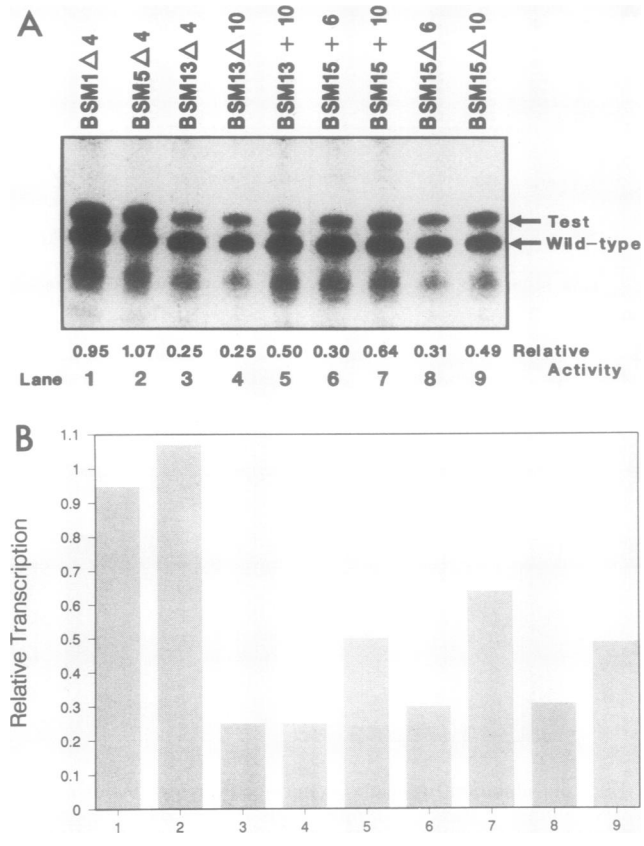

FIG. 9. In vitro analysis of the effects of distance-altering mutants at various sites of the rat rDNA promoter. The mutants indicated were cotranscribed with a wild-type template with unfractionated nuclear extracts. The mutant promoters were truncated to yield a 638-nt transcript, and the wild-type promoter was truncated to yield a 570-nt transcript. The parental constructs BSM 145/140 (BSM 1), BSM 123/118 (BSM 5), BSM 79/75 (BSM 13), and BSM 70/65 (BSM 15) are transcribed 0.9-, 1.0-, 1.87-, and 1.14-fold, respectively, as is the wild-type promoter under these same conditions (44).

binding of both mouse TIF-IB (8) and mouse SL-1 (5) to the promoter, whereas the substitution of adenine for guanine at −7 had no effect on the binding of TIF-1B or SL-1. Thus, the guanines at −16 in the vertebrate promoters are probably components of one domain of the CPE, presumably the SL-1 binding site. If the guanine at −7 is part of the SL-1 binding domain, it is not critical for binding but may be important for SL-1 to activate transcription. On the other hand, the G at −7 may be part of the site required for the binding of a third factor or even RNA polymerase I. It is interesting to note that the rat and mouse spacer promoters contain domains identical or highly homologous to the bases from −18 to −6 of the respective 45S promoters, which would include these two guanines (6, 7).

The amount of transcription supported by BSM 106/101,-16A/G was essentially the same as the promoter activity of the point mutant prR-16A/G in the in vivo assays. Although BSM 106/101 did not respond to the addition of UBF in in vitro transcription assays (33) and significantly weakened the binding of UBF to the promoter in both DNase footprinting assays and Southwestern (DNA-protein) blot analysis, it did not preclude binding in those assays and filter-binding assays (29). Thus, it would seem that in vitro assays with fractionated extracts did not yield a totally accurate depiction of the role of these nucleotides in a complete transcription system or in vivo. On the other hand, BSM 129/124,-16A/G was inactive in both the in vitro rescue assays and the in vivo assay. Two lines of evidence suggest that BSM 129/124 may be acting by weakening the binding of SL-1 to the UPE: (i) the SL-1 footprint in the UPE includes these nucleotides, and (ii) this same mutant had no effect on the binding of UBF to the promoter. Thus, it would seem that the effect of the UPE in both the in vitro rescue assays and the transfection experiments is more likely to reflect mutations in the smaller SL-1-binding site in the UPE than hexanucleotide substitutions within the larger UBF-binding domain. This suggests that a more complete mutagenesis of the UBF-binding site in the UPE, one which has a more quantitative effect on the binding of UBF, is required to more definitively demonstrate the role of UBF in transcription in vivo.

These results do not preclude the possibility that the UBF rescue assays reflect a weak or secondary UBF-binding site within the CPE of the rat rDNA promoter, similar to that reported for the human promoter. However, the results obtained with BSM 129/124, prR-16A/G, and the bidomain mutant BSM 129/124,-16A/G are most consistent with the hypothesis that the rescue assay reflects an interaction of UBF with the UPE which then results in the stabilization of the interactions occurring at the CPE.

An alternative method to mapping the *cis*-acting elements of the rat rDNA promoter by substitution mutagenesis, and one that would allow us to examine possible interactions between these domains, was to create distance-altering

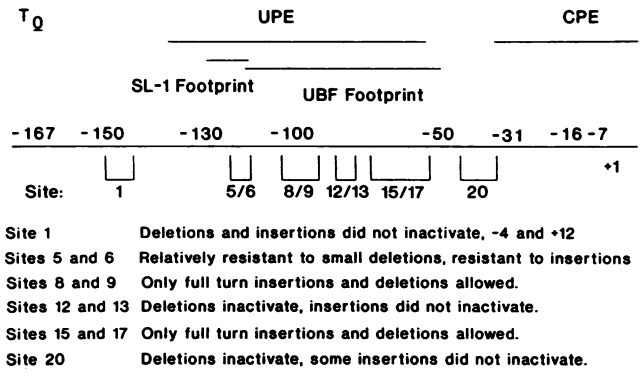

FIG. 10. Summary of the results of the studies of the distance-altering mutants. The results of these studies are portrayed along with observed and deduced binding sites of UBF and SL-1 on the rat rDNA promoter (37).

mutants at various sites within the promoter. Sites were chosen that would lie between putative transcription control domains as well as within them (summarized in Fig. 10). For example, in vitro transcription experiments suggested that the 5′ end of the UPE of the rat rDNA promoter lies circa −140 (5, 37). Thus, distance-altering mutants between −167 (T_0) and −140 would allow us to examine the question of the role of T_0 in transcription, as has recently been done for the *Xenopus laevis* promoter (27). Other sites, such as those between −50 and −60, lay within what our in vivo and in vitro experiments mapped as a region between the CPE and UPE, and the construction of distance-altering mutants within this domain would allow us to determine whether the relationship between the UPE and CPE was phased.

This study of distance-altering mutation across the rat rDNA promoter provided some interesting results. Although the promoter is relatively resistant to small (6-bp) substitutions, it demonstrated a widely variable sensitivity to deletions or insertions across most of its length (−147 to −40). Small deletions (up to 6 bp) and insertions of up to 17 bp had relatively small effects on promoter activity when they were placed between −160 and −110. However, larger deletions were prohibited (e.g., BSM 5Δ10 and BSM 5Δ16), probably because of the removal of *cis*-acting elements or because they moved two *cis*-acting elements too close together (binding interference). In contrast, small deletions further downstream, between −110 and −40, resulted in promoters that were significantly weakened. At sites 8 and 9, between −106 and −95, the inactivation that resulted from a 4-bp deletion was probably due to the disruption of the stereospecific alignment of the promoter domains. In this region, the insertion of 5 bp also inactivated the promoter, while the deletion or insertion of 9 or 11 bp restored promoter activity. The inactivation resulting from the deletion of more than 2 bp at site 13 (circa −79 to −75) was probably due to the removal of *cis*-acting elements or to binding interference.

Two regions, defined by sites 8 and 9 and by sites 15 and 17, demonstrated a phased response to distance-altering mutations. We have insufficient data with which to explain the effects of the mutations introduced at sites 8 and 9, i.e., between −106 and −95, although preliminary results suggest that this site may lie between two UBF-binding domains (30).

The cyclical behavior of the rDNA promoter activity in relation to the insertion or deletion of approximately half or full helical turns at sites 15 and 17, i.e., between −70 and −61, strongly suggests the phased interaction between proteins or protein complexes bound upstream and downstream of these sites. This is in agreement with observations of the behavior of several prokaryotic promoters, e.g., the activation of *Escherichia coli* promoters such as *lac*, *galp1*, and *malT* promoters by the cyclic AMP (cAMP) receptor protein (CRP)-cAMP complex in response to carbon limitation (see reference 11). Although the CRP-cAMP complex can bind at various distances from the initiation sites of these promoters, it must bind on the same side of the DNA helix as does the RNA polymerase in order to activate transcription (11). For example, the insertion of half-helical turns between the CRP-cAMP binding site and the transcription initiation site of the *melR* promoter results in no activation. Insertion of integral helical turns between the CRP site and the initiation site results in activation.

Recently, Pape et al. reported the results of the effects of various distance-altering mutations on transcription of *X. laevis* rDNA by mouse S-100 extracts (31). They observed an interesting effect. Promoters bearing one-half-turn helical spacing changes (between −126 and −28) did not direct initiation at the site normally used by mouse extracts, i.e., −4 (+1 being the authentic *X. laevis* rDNA transcription initiation site). Rather, transcription from those mutants initiated at +1. The authors suggested that the results indicate that the choice of the "face of the DNA helix on which initiation occurs" is directed in a stereospecific manner by the alignment of the UPE and CPE but not the specific face. The authors further suggested that the binding of a mouse transcription factor (PC-D) to the UPE of the *X. laevis* promoter was directing the side of the DNA, within the CPE, to which the same factor binds. Such a model could also involve the interaction of one molecule with two widely separated domains, as has been demonstrated for TF-IIIB (12). There is an alternative explanation for the results reported by Pape et al. that is consistent with our results. In this model, mouse PC-D can bind weakly to either of two out-of-phase positions within the CPE of the *X. laevis* promoter. A second factor (second molecule of PC-D) would then bind more strongly to the UPE and interact with the first, stabilizing its interaction with the CPE. Thus, as the stereospecific alignment of the binding site of the second factor is altered with regard to the possible binding sites of the first, a complex is formed that reflects this process and that directs initiation on one or the other face of the DNA.

Haltiner-Jones et al. (16) have reported that linker-scanning mutants of the human rDNA promoter with small deletions within the UPE (their constructs were designated LS −86/−73 and LS −107/−94) function as well as do wild-type promoters but that larger deletions (≥16 bp) reduce transcription by 95%. They did not report a change in the initiation site. While their results are consistent with an interaction between the complexes formed on the UPE and CPE, they did not report a stereospecific relationship between the UPE complex and the transcription initiation site.

The behaviors of the distance-altering mutants constructed at sites 15 and 17, and possibly those at sites 8 and 9, indicate a stereospecific relationship between the UPE and the CPE, and possibly within the UPE. However, we have not observed the utilization of an alternative transcription initiation site. Thus, the paradigm proposed for the transcription of *Xenopus* rDNA by mouse extracts does not explain our results. While it is true that the UPE is not essential for in vitro transcription of the human, mouse, and rat rDNA promoters, it is essential in vivo (16, 19). Further, we have constructed point mutants of the CPE whose

transcription requires an intact UPE (37, 44) in vivo, and the results reported herein demonstrate an interaction between the UPE and the CPE in vitro as well. Thus, while it is apparent that the interaction between the UPE and CPE can be demonstrated to be stereospecific, at least for the rat rDNA promoter, our results indicate that the transcription initiation site is dictated not by the UPE but by the CPE.

We have previously reported that rat SL-1 binds both to the UPE and to the CPE of the rat 45S and spacer rDNA promoters and is essential for transcription (in vitro) from deletion mutants of the 45S promoter that lack the UPE (37, 38). We would suggest that when rat SL-1 (either alone or in combination with another factor) binds to the CPE, it behaves analogously to the *Acanthamoeba* rDNA transcription initiation factor (TIF-1B) and directs initiation at a fixed distance (21). The protein complex bound to the UPE stabilizes the binding of the core complex, thus increasing the efficiency of the transcription.

Finally, while we have not forthrightly said so, the results presented herein are consistent with a model in which the functional rDNA promoter is bent or looped, with at least one bend centered circa −70 to −61 and a second possible bend centered circa −106 to −95. While such a model would be consistent with both our results and those of Pape et al. (31), other physicobiochemical lines of evidence are required to confirm such bending.

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