Interaction of RNA polymerase I transcription factors with a promoter in the nontranscribed spacer of rat ribosomal DNA

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ABSTRACT

The spacer promoter of the rat rDNA repeat consists of two functional domains: a core (proximal) element that is sufficient for transcription *in vitro*, and an upstream (distal) promoter element that increases the efficiency of transcription. Two of the transcription factors that interact with the 45S promoter also interact with the spacer promoter. Rat SL-1, is required for transcription of the spacer promoter by heterologous extracts, e.g. human, and rat SF-1 is required for efficient transcription *in vitro*. Order-of-addition experiments demonstrated that the preinitiation complex formed by these factors on the spacer promotor is not as stable as the complex formed on the 45S promoter. DNase 1 footprinting experiments demonstrated binding sites for rat SL-1 and SF-1 on the distal element of the spacer promoter. The topology of the domains of the spacer promoter may explain both the reduced stability of the preinitiation complex formed on that promoter and the lower efficiency of transcription of that promoter when compared to the 45S promoter.

INTRODUCTION

Eukaryotic ribosomal RNA genes (rDNA) are organized as tandem repeats. Each repeat contains a transcribed or coding region, which codes for the ribosomal RNA precursor, e.g. 45S rRNA, and a non-coding region, the nontranscribed spacer (NTS) (1,2). In most vertebrates, the region within 200 bp of the transcription initiation site (+1 being the first nucleotide of the pre-rRNA) contains the promoter that directs the synthesis of pre-ribosomal RNA. In mammals this 'major' promoter consists of at least two interacting elements referred to as the core promoter element (CPE) and the upstream promoter element (UPE). The CPE (−31 to +6) is necessary and sufficient for transcription *in vitro* and is required for transcription *in vivo*. The UPE is required for efficient transcription *in vivo* and *in vitro* and for stable complex formation *in vitro* (3−16).

RNA polymerase I enhancer elements have been identified in the nontranscribed spacers of yeast (26), *Xenopus* (27−30) and *Drosophila* (31,32). Several potential enhancer elements have been demonstrated (33,34) in the NTS of the rat rDNA repeat, including one between −286 and −1018 (33) (+1 being the first nucleotide of 45S pre-rRNA). Additional RNA polymerase I promoters, referred to as spacer promoters, have been found in the nontranscribed spacers of *Drosophila* (31,35), *Xenopus* (27), rats (36), mice (37), and Chinese hamsters (51). An element that functions as a promoter *in vitro* has been identified in the NTS of some yeast strains (38). We demonstrated that the region of the rat rDNA NTS circa −713 contains an RNA polymerase I promoter that has a 13 bp sequence identity to the 45S promoter (36). Similar elements, also with low amounts of sequence identify with their respective 45S promoters, were found in the spacers of the mouse and CHO rDNA repeats (37).

In addition to RNA polymerase I, two transcription factors have been identified in rat and human cells; the human factors are referred to as human SL-1 and UBF-1 (17−19), and the rat factors as rat SL-1 and SF-1 (Smith, *et al.*, manuscript submitted). Rat SF-1 and human UBF-1 interact with the upstream elements of their respective promoters, and stimulate transcription *in vitro* (17, Smith *et al.*, manuscript submitted). Recent experiments suggest that rat SF-1, human UBF-1 and Xenopus UBF may be homologous proteins (Pikaard *et al.*, unpublished observation).

Transcription terminators are located immediately 5′ and 3′ of the transcribed region (20,21). Those terminator elements that are proximal to the 3′ terminus of 28S rRNA either direct the termination of rRNA synthesis, as in the mammalian repeats, or are sites of rapid RNA processing, as in *Xenopus* (8). The terminator element proximal to the 5′ end of the transcribed region (referred to as T_0 in the mammalian rDNA repeats or as T_3 in the *Xenopus* rDNA repeat) may act as an element of the adjacent promoter (20,22,23). In contrast to the complex organization of the vertebrate rDNA promoters, the rDNA promoter of Acanthamoeba consists of a single element (24,25).

Transcription of the *Xenopus* spacer promoters has been visualized by electron microscopy and in run-on transcription experiments (39). Transcription of part of the spacer region of the mouse and rat ribosomal RNA genes has been demonstrated in nuclear run-on experiments (40,37). Other studies failed to detect steady-state levels of such transcripts, which led to the

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conclusion that the half-lives of the spacer transcripts are very short. The spacer transcripts and read through transcripts of the spacer in *Xenopus laevis* are also very rapidly degraded *in vivo* (39, 21).

Transcription of the spacer promoter may be physiologically significant. Transcription of the spacer promoter might direct an activated form of the polymerase toward the major promoter, acting to increase the local concentration of polymerase, and increasing the frequency of initiation. This mechanism of enhancement has been termed 'readthrough enhancement' (29,27). In this mechanism the transcripts themselves would serve no purpose and they could be rapidly degraded after the complexes reach the promoter. In nuclear run-on experiments Harrington and Chikaraishi detected nascent transcripts that originated in the NTS and terminated near the 'Sal 1 box' or T_0, 167 bp upstream of the transcription initiation site of rat pre-ribosomal RNA (40). In a second model, spacer promoters could function as staging areas where transcription factors and/or RNA polymerase I could associate to form a 'preinitiation complex' followed by the transfer of that complex to the major promoter.

Although the mechanism of action of enhancers has not been elucidated, it appears that they can act as 'distal' promoter elements, and in fact, can be shown to interact with some of the same proteins that bind to the proximal promoter elements (42). In this case, it is possible that merely the assembly of the preinitiation complex in the spacer could be responsible for the activation of the pre-ribosomal RNA promoter. Thus, a logical beginning in dissecting the action of the spacer promoters would be to determine: 1) which transcription factors interact with them, 2) if there are factors that interact with both the spacer and the pre-ribosomal RNA promoters, and 3) the precise sequence in the spacer promoter to which these proteins bind. As the spacer promoters represent 'natural mutants' of the precursor promoters, these studies should allow us to determine the important topological relationships of the promoter elements.

In this manuscript, we report that, like the rat pre-ribosomal RNA promoter, the spacer promoter of the rat rDNA repeat appears to consist of two functional domains, a core promoter that is sufficient for a low level of transcription *in vitro* and an upstream domain increases the eficiency of transcription. Besides RNA polymerase I, two transcription factors, SL-1 and SF-1 interact with the spacer promoter. The same highly purified factor, rat SL-1, that programs human RNA polymerase I to transcribe the rat 45S promoter can also program HeLa cell extracts to transcribe the spacer promoter. The same factor that stimulated the *in vitro* transcription of the rat 45S promoter (referred to as SF-1) also stimulated transcription from the spacer promoter. Footprinting experiments demonstrated that the two transcription factors, SF-1 and SL-1, bind to analogous sites in the upstream promoter elements of both the spacer and the 45S promoters. The distance of the UPE of the spacer promoter from its CPE may explain why transcription from the spacer promoter is less efficient than is transcription from the 45S promoter, and why the spacer promoter does not sequester transcription factors as efficiently as does the 45S promoter.

MATERIALS AND METHODS

Plasmid DNA

The basic templates used in this study are shown in Figure 1. All of the inserts were cloned in pUC 19. Most of these clones have been described previously (33,36,43). Plasmid puTH was

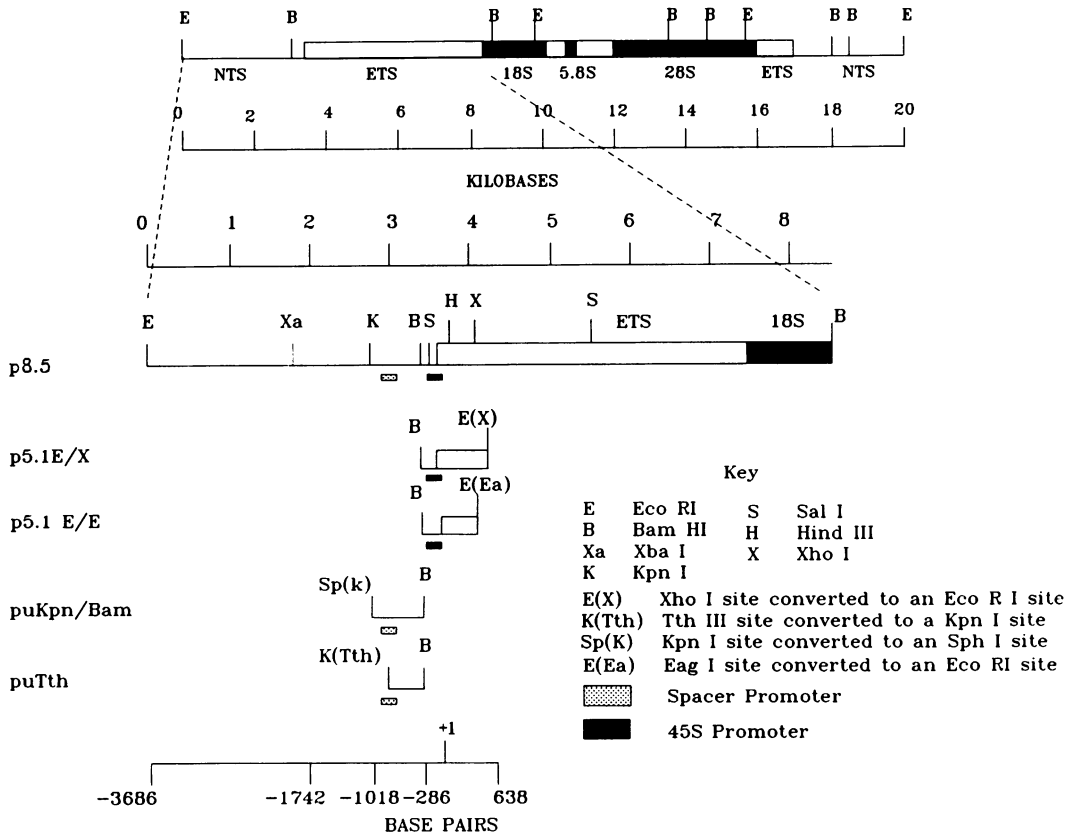

Figure 1. Schematic representation of the constructs used in this study. The constructs are as described in the key.

derived from puKpn/Bam. The unique Tth lll site in the insert was converted to a Kpn l site by the addition of Kpn l linkers, and then the plasmid was digested with Kpn l and religated. The result of this manipulation was the deletion of the sequences 5′ of −52 (+1 being the *spacer* transcription initiation site). The deletion mutants −192, −143, −90 and −67 were derived by the polymerase chain reaction using 5′primers that defined the 5′boundaries of the deletions and contained Kpn 1 sites. The identities of the deletion mutants were confirmed by sequencing through the new Kpn l sites (44). The fragment used for footprinting was derived from pUCHF3 (16). That clone contains a 688 bp Hinfl fragment (nucleotides −1106 to −417) from the NTS that was subcloned into the Sma l site of pUC12.

Isolation and fractionation of nuclear extracts

Nuclear extracts were prepared from Novikoff hepatoma ascites cells or from logarithmic cultures of HeLa cells as described (45). After the final dialysis, the crude extracts were quick frozen and stored at −80°C. Subsequently, extracts were fractionated by DEAE-sephadex column chromatography as described previously. This yields a fraction, referred to as either DE-175 or as SF-1 depleted extract, that is capable of specific transcription and of forming the stable preinitiation complex (3), as well as a second fraction, DE-500 (45), that stimulates specific transcription by RNA polymerase I. DE-175 contains rat SL-1 as well as RNA polymerase I, and is the fraction from which rat SL-1 was purified when necessary. The activity present in DE-500 responsible for the stimulation of transcription is referred to as SF-1 (Stimulatory Factor-1). The purification of RNA polymerase I, SL-1 and SF-1 was as described (Smith *et al.*, manuscript submitted).

In vitro transcription

The conditions for *in vitro* transcription were described previously (3,33,36,43). A standard 50 μl reaction contained between 0.02 and 0.4 μg of truncated template and 1 μg of non-specific DNA, either pUC 18 or pBR322. When templates were preincubated with crude extracts or subfractions, the conditions of the preincubation are the same as in the final assay except that nucleotides are added after the preincubation. A constant amount of an end-labeled DNA fragment was added to the reaction mixture before the purification steps and served as an internal standard for the efficiency of recovery of nucleic acids. The synthesis of specific transcripts was visualized by autoradiography and quantitated by densitometry of linear exposures as described previously (33).

DNase 1 footprinting

DNase 1 footprinting was carried out essentially as described (46). The Bam/Sal fragment of pUCHF3 used for the footprinting experiments shown was labeled at the internal Sal I site (−650) and released by digestion with Eco RI, which cleaves in the vector. The top strand (noncoding) was labeled with Klenow enzyme and [α-^{32}P]ATP, and the bottom strand was labeled with polynucleotide kinase and [γ-^{32}P]ATP (47). After digestion with DNase, the fragments were extracted with organic solvents, ethanol precipitated, resuspended in 90% formamide and displayed by electrophoresis on 6% polyacrylamide-urea sequencing gels, followed by overnight autoradiography using an intensifying screen. Sequencing reactions (48) of the respective strands were electrophoresed alongside the products of the DNase digestion to allow for the alignment of the protected sites.

RESULTS

Rat SL-1 is Required for Transcription from the Rat Spacer Promoter by HeLa Extracts and Rat SF-1 Stimulates Transcription from the Rat Spacer Promoter

Transcription by RNA polymerase I is, with one reported exception (49), species-specific, i.e. extracts of primate cells will not transcribe rodent rDNA and vice versa. This selectivity has been attributed to a single transcription factor (10), which we refer to as rat SL-1. Furthermore, rat SL-1 interacts both with the core and upstream promoter elements of the 45S promoter, and is required for transcription of the 45S promoter (Smith *et al.*, manuscript submitted). Experiments were carried out to determine if rat SL-1 and rat SF-1 also interacted with the spacer promoter. Due to the low level of activity of the rat spacer promoter in the absence of SF-1, we were unable to demonstrate SL-1 dependent transcription from the CPE of the spacer promoter or from the intact spacer promoter using reconstitution experiments, unless SF-1 was added to the assays (data not shown). Instead, we made use of the ability of rat SL-1 to program the transcription of rat rRNA by HeLA cell nuclear extracts. Neither the rat 45S promoter, nor the rat spacer promoter were transcribed by HeLa cell nuclear extracts (Figure 2, lanes 1 and 5). However, when those extracts were supplemented with SL-1, both templates were transcribed (Figure 2, lanes 2 and 6). SF-1, which is not specifies-specific (manuscript in preparation) was not capable of programming the HeLa cell extracts (Figure 2, lanes 3 and 7), but could stimulate SL-1-dependent transcription from either template (Figure 2, lanes 4 and 8).

Efficient Transcription from the Spacer Promoter Requires SF-1 and an Upstream Promoter Element

In previous experiments (36) using an active subfraction (DE-175) of whole cell extracts, we reported that transcription of the spacer

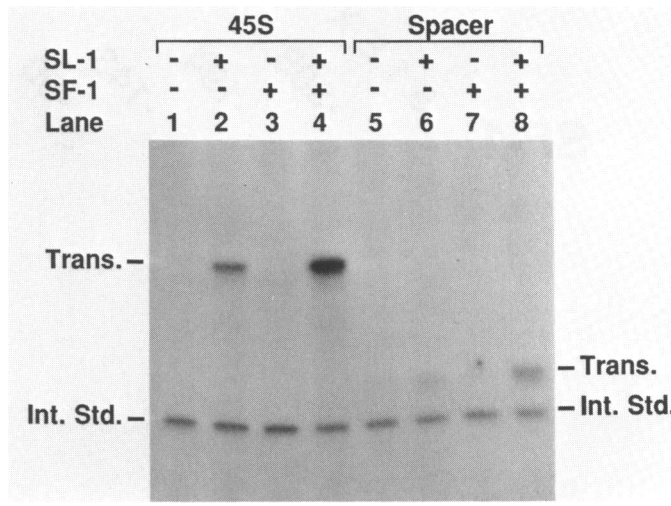

Figure 2. Transcription of the rat 45S and spacer promoters in the presence and absence of highly purified forms of SL-1 and SF-1 by heterologous extract. Equal amounts (0.2ug) of the 45S (lanes 1−4) and spacer promoter (lanes 5−8) were transcribed by HeLa extracts supplemented with no rat factors (lanes 1 and 5), rat SL-1 alone (lanes 2 and 6), rat SF-1 alone (lanes 3 and 7), or both rat SF-1 and rat SL-1 (lanes 4 and 8). The 45S promoter was truncated to yield a 638 nt transcript, and the spacer promoter was truncated to yield a 427 nt transcript. A constant amount of an internal standard (Int. Std.) was added for the recovery of nucleic acids. A lighter exposure of the same autoradiograph was scanned with a BioRad video densitometer for quantitation.

promoter was only 10% as efficient as transcription from the 45S promoter *in vitro*. We have repeated those experiments using unfractionated nuclear extracts, and with the equivalent subfraction (DE-175) of the nuclear extracts (Figure 3). When the spacer and 45S promoters were transcribed with unfractionated nuclear extract, the 45S promoter was only 30% more efficient than was the spacer promoter (Figure 3A). The difference between the two promoters was greatly accentuated when both templates were transcribed with 'SF-1 depleted extract', (Figure 3C). The 500 mM $(NH_4)_2SO_4$ wash of the

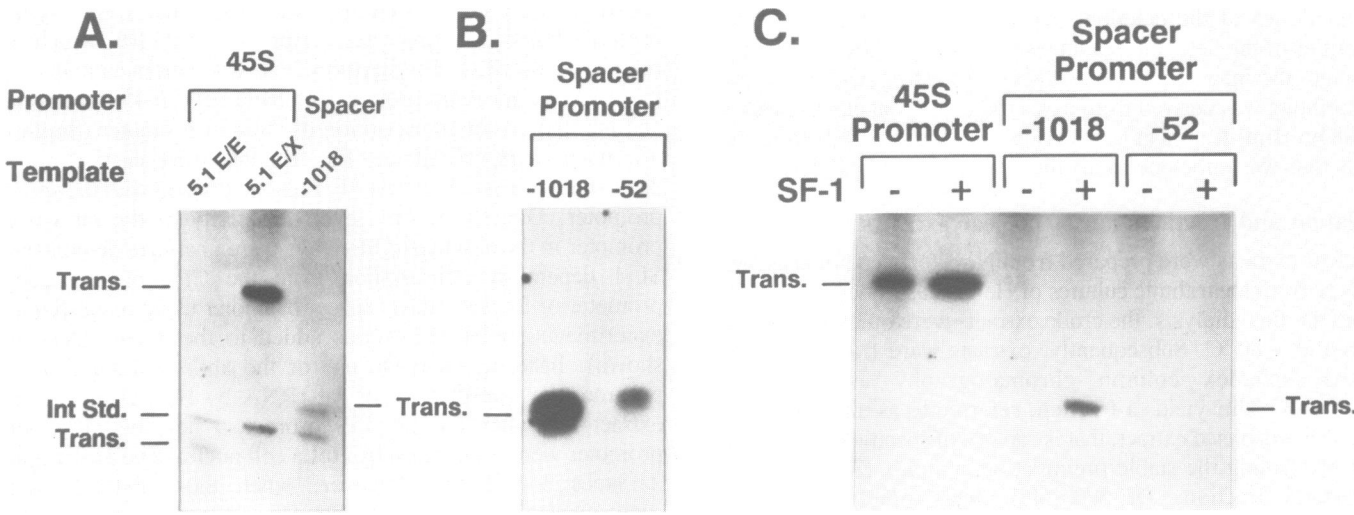

Figure 3. Transcription assays utilizing intact nuclear extracts, extracts depleted of SF-1, and SF-1 depleted extracts supplemented with SF-1 demonstrate that the spacer promoter consists of more than one domain. A. Transcription of intact 45S and spacer promoters with unfractionated nuclear extracts. p5.1 E/E is a 45S promoter truncated to yield a 330 nt transcript, in contrast to the 638 nt transcript from p5.1 E/X. The spacer promoter template −1018, was truncated to yield a 427 nt transcript. B. Transcription of the intact spacer promoter and a spacer promoter construct deleted to −52 (+1 being the site of transcription initiation in the spacer) with intact nuclear extract. C. Transcription of the 45S promoter, the intact spacer promoter and the −52 deletion of the spacer promoter with SF-1 depleted nuclear extracts without (−) or supplemented (+) with SF-1. At these template levels (0.1 μg/assay) only transcription from the 45S promoter is observed in the absence of SF-1.

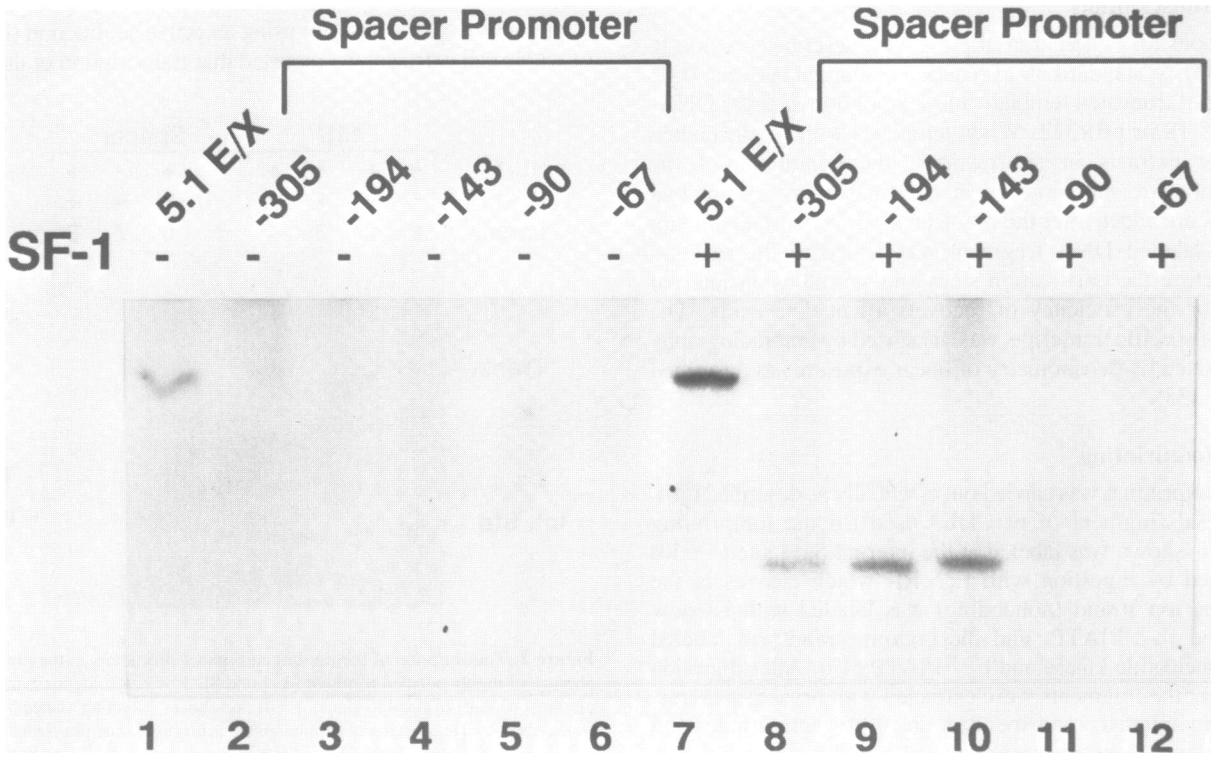

Figure 4. Transcription of 5′ deletion mutants of the spacer promoter maps the 5′ boundary of the domain required for the response to SF-1 to between −143 and −90. In lanes 1−6 the indicated templates were transcribed by SF-1 depleted extract. In lanes 7−12 the extracts were supplemented with CM-Sephadex purified SF-1. In the lanes where the templates were derived from the spacer promoter, the numbers indicate the 5′ boundary of the rat sequences, numbered relative to the transcription start site in the spacer.

DEAE-Sephadex column provided a fraction (SF-1) that significantly stimulated transcription from the spacer promoter (Figure 3C).

We found that the effect of SF-1 on transcription from the 45S promoter, required an intact UPE in cis with the CPE (Smith *et al.*, manuscript submitted). Therefore, we sought to determine if the spacer promoter also consisted of multiple domains.

We constructed a −52 (+1 being the transcription initiation site of the spacer promoter) deletion mutants of the spacer promoter (puTh or pTh/Bam) to examine the possibility that the spacer promoter also consisted of multiple domains. This template was transcribed by intact nuclear extract (Figure 3B), but transcription from −52 did not respond to the addition of SF-1 to a depleted extract (Figure 3C), and it was transcribed by an unfractionated extract at a much lower efficiency than was −1018 (Figure 3B). These experiments demonstrated that the region within 52 bp of +1 were sufficient for the spacer promoter to function in transcription, and suggested that a second domain was required for the response to SF-1. Subsequent mutagenesis and transcription experiments demonstrated that the 5′ boundary of the domain required for the spacer promoter to respond to SF-1 was between −143 and −90 (Figure 4).

Thus, like the 45S promoter, the spacer promoter appears to consist of a core promoter element, which is sufficient for transcription *in vitro*, and an upstream promoter element, which is required for more efficient transcription. These observations are consistent with the results of DNase I footprinting experiments that demonstrated an SF-1 binding site 90 bp upstream of the transcription initiation site of the spacer promoter (see below).

The stimulation of transcription from both the 45S and spacer promoters by SF-1 suggested that the same factor (SF-1) interacted with both promoters. However, the possibility exists that the stimulation of transcription from the two promoters was the effect of two different activities in the chromatographic fraction that contained SF-1.

In order to determine if the same factor was responsible for stimulating transcription from both the spacer and 45S promoters, we took advantage of the observation made with the 45S promoter that SF-1 would commit to a template in the presence of the other transcription factors and RNA polymerase I. As shown in Figure 5 (lanes 1−4), both the spacer and the 45S promoters responded to the addition of SF-1, when SF-1 was present during the preincubation step. In lanes 1−4, no second template was added. When SF-1 was present during the preincubation of the 45S promoter, but not present in the preincubation of the spacer promoter, and the templates were mixed prior to the addition of nucleotides, only transcription from the 45S promoter was affected (Figure 5, lanes 6 and 9). When SF-1 was added to the preincubation of the spacer promoter, but not to the preincubation of the 45S promoter, transcription from the spacer promoter was stimulated, and so was transcription from the 45S promoter (Figure 5, lanes 7 and 10). When SF-1 was present in both preincubations, transcription from both templates was stimulated (Figure 5, lane 11).

The results of the assays in which SF-1 was preincubated with 45S promoter indicated that both the 45S and spacer promoters were utilizing the same factor, and that once this factor was sequestered by the 45S promoter, it was not free to exchange to the spacer promoter,e.g. Figure 5, lane 6. However, the interpretation of the results of the assays in which SF-1 was preincubated with the spacer promoter prior to mixing were not clear cut. Although it was apparent that transcription from the spacer promoter was stimulated in those experiments, so was transcription from the 45S promoter, (compare lanes 5 and 7 in Figure 5). Furthermore, the degree of stimulation of transcription from either promoter in lane 7 was less than that observed when each was preincubated individually with SF-1 (lanes 2 and 4). This suggested that SF-1 was not binding stably with the spacer promoter, and that it could exchange with the 45S promoter after the two promoters were mixed. When SF-1 was present in both preincubations, transcription of both promoters, particularly the spacer, was stimulated (compare lanes 10 and 11 Figure 5). It is plausible that the inclusion of SF-1 in both preincubation tubes, prior to mixing, resulted in increased levels of transcription from

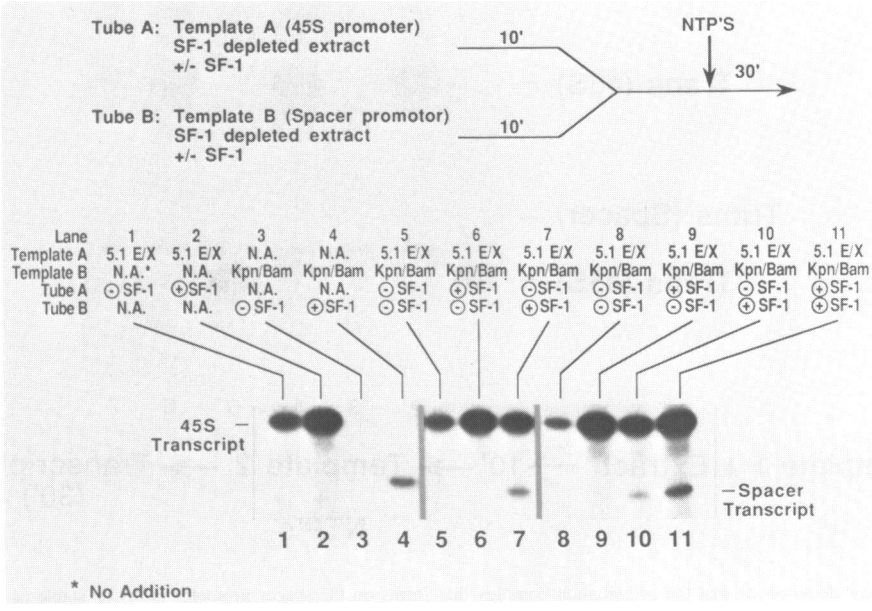

Figure 5. Factor sequestration assays demonstrate that the spacer promoter is utilizing the same factor (SF-1) as is the 45S promoter. The assays were carried out as indicated in the schematic. At the template levels used (0.05 μg/assay) transcription from the spacer promoter is virtually undetectable unless SF-1 is added (compare lanes 3 and 4).

both templates as this allowed for saturation of the SF-1 sites on the 45S promoter before the templates were mixed.

We have observed that the spacer promoter does not form a stable preinitiation complex (see below). This suggests that we may be observing the exchange of SF-1 and the remainder of the transcription machinery from the spacer promoter to the 45S promoter in the experiments described above, but not vice versa.

The Preinitiation Complex that Forms on the Spacer Promoter is a Metastable Complex

Our results indicated that the 45S and spacer promoters utilized two of the same transcription factors (SF-1 and SL-1). However, the SF-1 sequestration experiments indicated that the binding of SF-1 to the spacer promoter was not as strong as the interaction between SF-1 and the 45S promoter. We have found that the formation of a stable complex of SF-1 and the 45S promoter was dependent upon the presence of other transcription factors. Therefore, we sought to determine if the spacer promoter supported the formation of a stable, SF-1 independent, preinitiation complex.

In these experiments, one template was preincubated with SF-1 depleted extract at room temperature, allowing for the formation of a stable preinitiation complex, then a second template was added along with NTPs, and transcription was allowed to proceed at 30°C. If a stable preinitiation complex formed on the first template, and the level of template added was sufficient to sequester all of the factors, then transcription from the second template would be undetectable. To establish the conditions for this experiment, two wild-type 45S promoters (distinguished by the sites of truncation) were competed against one another (Figure 6, lanes 4 and 5). Preincubation of the SF-1 depleted extract with either p5.1E/X or p5.1E/E prevented the transcription of the second 45S promoter. When extract was preincubated with p5.1E/X, there was no detectable transcription from the spacer promoter, puKpn/Bam (Figure 6, lane 6). However, when the spacer promoter was the first template, a low level of transcription from p5.1E/X was observed (Figure 6, lane 7). This would not be the predicted result if the spacer promoter had sequestered the transcription factors in the same manner as did the 45S promoter.

These results are similar to observations reported on the inability of deletion mutants of the 45S promoter to form stable complexes (3), and suggest that the preinitiation complexes that form on the spacer promoter are not stable preinitiation complexes, but are metastable. These results would also explain why we observed what might have been an exchange of at least SF-1 from the spacer promoter to the 45S promoter.

Rat SL-1 and SF-1 Protect Two Domains of the UPE of the Spacer Promoter from Digestion by DNase I

The experiments with the deletion mutants indicated that sequences 5′ of −90 were required for SF-1 to stimulate transcription from the spacer promoter. We have found that both SF-1 and SL-1 interact with the UPE of the rat 45S promoter. These observations suggested that DNase I footprinting experiments would demonstrate that SL-1 and SF-1 were binding 5′ of −90, and might also provide evidence relevant to understanding the observed differences in the efficiency of template utilization. Footprinting experiments indicate that SF-1 protects a 40 bp region of the spacer promoter (−91 to −132 relative to the spacer initiation site) and SL-1 protects a 25 bp region just upstream of the SF-1 site (−165 to −187) (Figure 7).

When the domains of the spacer and 45S promoters protected by SF-1 were compared a conserved sequence was found (Figure 8).

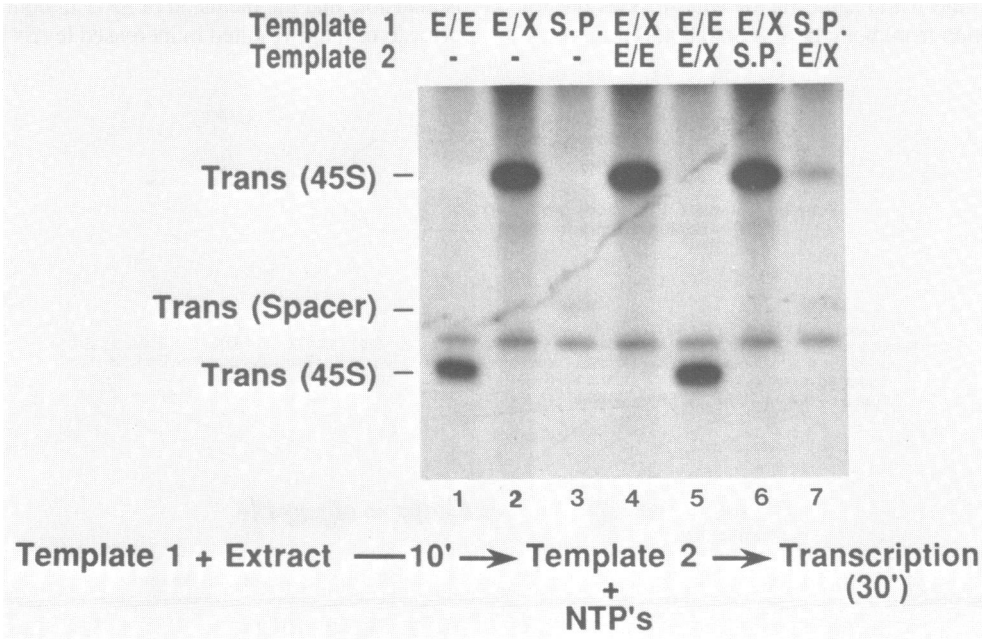

Figure 6. Order-of-addition assays demonstrate that the preinitiation complex that forms on the spacer promoter is not as stable as that which forms on the 45S promoter. In lanes 1,2, and 3 two wild-type 45S promoters (p5.1E/X and p5.1E/E) and one spacer promoter construct (puKpn/Bam) were transcribed individually. In lanes 4 and 5 the 45S promoter constructs were tested for their ability to form stable preinitiation complexes. In lanes 6 and 7 the 45S and spacer promoters were completed against one another. Preincubation of extract with either of the 45S promoters prevents transcription of a second template (lanes 4−6). Preincubation of the extract with the spacer promoter, did not prevent transcription of the 45S promoter (lane 7). Equal moles (0.1 pmol) of each template were added to each assay.

DISCUSSION

We have demonstrated that the spacer promoter of the rat rDNA repeat has several properties in common with the 45S promoter. However, it is consistently a weaker promoter, and it does not form a stable preinitiation complex. The spacer promoter interacts with two of the factors that interact with the 45S promoter. Transcription from the spacer and the 45S promoters is species specific, and the same highly purified factor, rat SL-1, directed HeLa extracts to transcribe both promoters. We have demonstrated that a second factor, rat SF-1, stimulates transcription from both promoters as well. These experiments have also been carried out using affinity purified SF-1, providing further evidence that the spacer and 45S promoters are interacting with the same factor. In conjunction with the studies on rat SF-1, we demonstrated that the spacer promoter consisted of two elements, a core element, and an upstream domain, that is required for the response to SF-1. The core promoter is sufficient for a very low level of transcription and the upstream promoter element is required for a more efficient level of transcription.

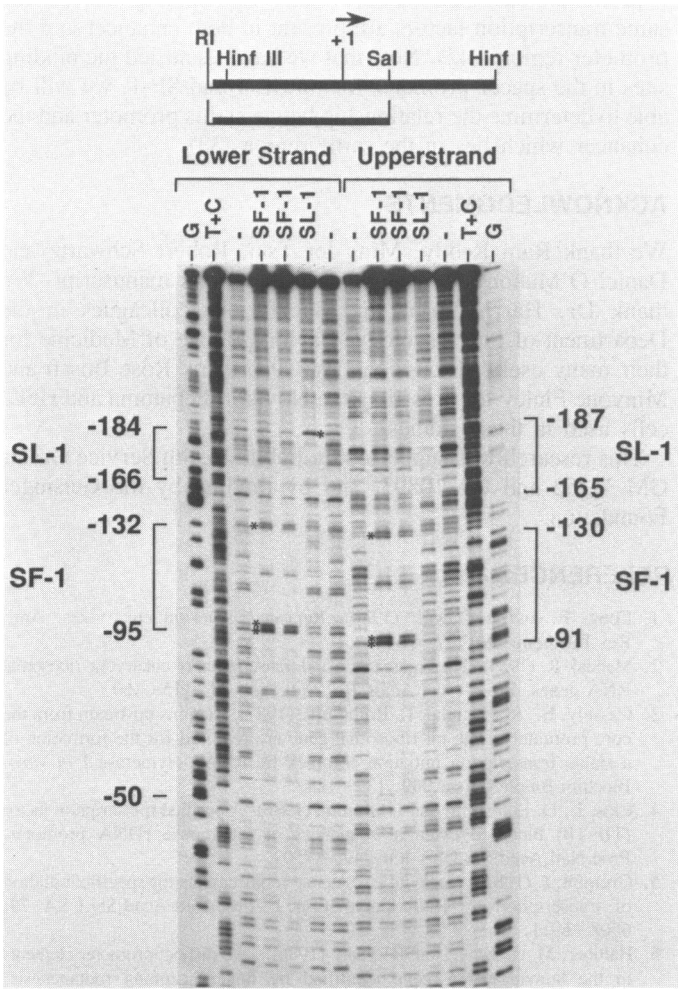

Figure 7. Localization of the rat SF-1 and rat SL-1 binding sites on the rat spacer promoter using DNase I. Highly purified and concentrated forms of rat SL-1 and SF-1 were incubated with end-labeled fragments of the spacer promoter, the presence of excess unlabeled pUC 18, under standard transcription conditions. Footprinting with DNase I was carried out as described in 'Material and Methods'. The fragment used was an Eco RI/Sal I digested fragment that contained the spacer promoter as indicated (+1 indicates nucleotide one of the spacer transcript, and the arrow indicates the direction of transcription). The fragment was labeled at the Sal I site by the action of either Klenow fragment (upper strand) or polynucleotide kinase (lower strand). Chemical sequencing reactions were run alongside the DNase digestions to allow for the correct alignment of the footprints.

Our studies on the 45S promoter suggest that when both factors are bound to their respective elements in the UPE, there may be a protein-protein interaction required for the stimulation of transcription (Smith *et al.*, manuscript submitted). A similar mechanism has been proposed for the interactions of human SL-1 and human UBF-1 (17,19). The inability of SF-1 to stimulate transcription of the −90 and −52 deletions of the rat spacer promoter suggested that at least the 5′ boundary of the SF-1 binding site lay upstream of −90. This was confirmed with DNase 1 footprinting. The SF-1 and SL-1 footprints would indicate that the binding sites of the two factors within the UPE of the spacer promoter are adjacent, with the 5′ border SF-1 footprint beginning 40 bp downstream of the SL-1 footprint. The SF-1 footprint on the rat 45S promoter also lies downstream of the SL-1 footprint. Thus, the overall arrangement of the cis-acting elements of the spacer promoter is similar to that of the 45S promoter (3,6,50). However, the experiments with the deletion mutants (Figure 4) would indicate that the SL-1 binding site defined by DNAse I in the UPE of the spacer promoter is not required for the effect of SF-1. Thus, the biochemical significance of this site is questionable, until we better understand the interaction of SL-1 and SF-1, and SL-1 with the promoters.

The structural differences between the two promoters may explain why the spacer promoter is less efficiently transcribed *in vitro*, and why it does not support the assembly of a stable preinitiation complex. For example, the SF-1 binding site is displaced approximately 40 bp upstream in the spacer promoter (relative to the initiation site) compared to its location in the 45S promoter. This extra four turns of the helix might effect the interaction between the UPE and the CPE in the spacer promoter. In this regard, Haltiner *et al.* (6) demonstrated that distance mutations in analogous sections of the human 45S promoter were down mutations.

Tower *et al.* (51) recently characterized the spacer promoters of the mouse and Chinese hamster ribosomal RNA genes. In contrast to our data for the rat spacer promoter, the spacer promoter of the hamster rDNA repeat was as active as the hamster 45S promoter and was capable of forming a stable preinitiation complex. However, the mouse spacer promoter was less active than the mouse 45S promoter and was not found to bind transcription factors.

We have indirect evidence that the requirement for SF-1 for transcription from the spacer promoter might be attributable to the instability of the preincubation complex that forms at the core element of the spacer promoter. 45S promoters with weakened core promoter elements were inactive in the absence of SF-1 and rescued by the addition of SF-1 (Smith *et al.*, manuscript submitted). Thus, weak interactions over the core promoter element can be strengthened by the addition of SF-1. It is possible that the effect of moving the SF-1 binding site as far upstream from the core promoter element, as they are in the spacer promoter, reduces the stabilizing effect of the complex that forms on UPE on the interactions of SL-1 and RNA polymerase I on the CPE. This is currently under investigation.

Comparison of the sequences of the SF-1 binding sites in the spacer and the 45S promoters revealed two nucleotide homologies; one internal and the other at the 5′ border of each SF-1 footprint (Figure 8). The border sequence is 91% identical. The internal sequence (9 bp) is 88% identical, and sequences similar to this sequence are found in the promoters of the mouse

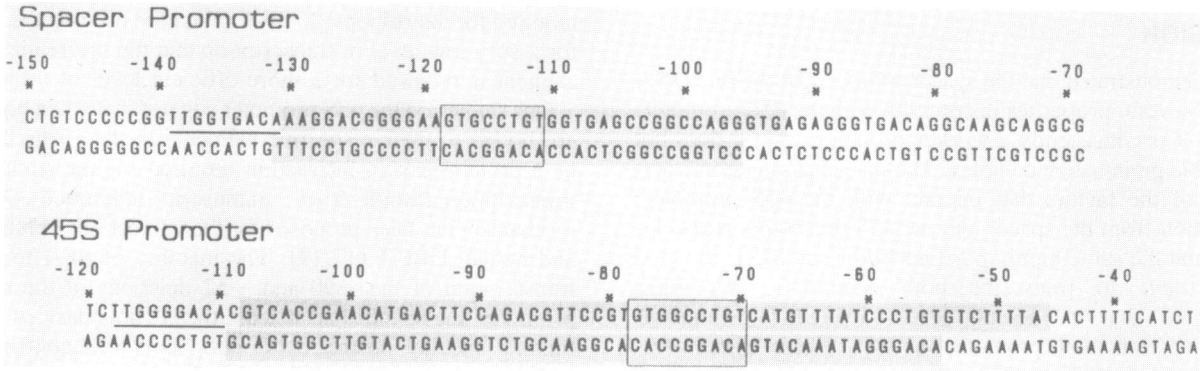

Figure 8. Comparison of the sites on the 45S and spacer promoters protected by SF-1. The footprint of SF-1 and the 45S promoter is submitted for publication. The nucleotides protected in the footprinting reactions are shaded. A consensus SF-1 recognition sequence within the protected regions is indicated by the boxed nucleotides. A secondary homology, that lies just outside of the footprints, is indicated by the thick line.

and human 45S genes. Based on the results of these footprinting experiments, oligonucleotide affinity columns were constructed using either the spacer promoter or the 45S promoter sequences protected by SF-1. Affinity purification of SF-1 over either column resulted in equivalent preparations (Smith *et al.*, manuscript in preparation).

Attempts to determine the role of spacer promoters in the transcription of ribosomal RNA have yielded conflicting data. Whereas several studies have suggested (29,30) that the repeating 60/81 bp elements of the *Xenopus* NTS are fully functional enhancers, other have reported (27,28) that constructs with functional 40S promoters in cis with inefficient spacer promoters failed to compete with genes with wild type spacer promoters and were transcribed 20 fold less (27,28). There do not appear to be multiple repeats of 'promoter-like' elements in the spacers of the yeast, mouse and rat rDNA repeats that would correspond to the *Xenopus* 60/81 repeats. (Although the rodent rDNA repeats contain variable numbers of 115 bp elements). However, the spacers of all three species have at least one promoter or promoter-like element. It would seem likely that the enhancer activity of the spacer 'promoters' is not dependent upon transcription because: 1) stimulation of transcription by spacer elements has been shown to be at least partially independent of orientation, and 2) some copies of the yeast enhancer do not support transcription *in vitro* and none have been shown to support transcription *in vivo*. It would seem unlikely that the spacer promoters represent a series of fortuitous mutations, but it is possible that they are the remains of promoter duplications (created by crossover events) which are advantageous and have been maintained. We have shown that the spacer promoter of the rat rDNA repeat is not a single element, and that it appears to have an array for cis-acting elements which interact with the same transcription factors as the 45S promoter.

Our observations do not discriminate between two additional models for how RNA polymerase I enhancers may function. They are consistent with a model in which metastable complexes would form on the spacer promoter and then 'transfer' to a more stable interaction with the 45S promoter. In a second model, the transcription factors that bound to the spacer promoter would also interact with the 45S promoter, either with the DNA directly or with the complexes forming there. In this way, the interaction between the spacer promoter and the 45S promoter would result in a quantitative or qualitative change in the formation of the transcription complexes that form on the 45S promoter. This model would not require transcription from the spacer promoter. It resembles one of the models proposed for RNA polymerase II promoters based on the observation that binding sites for the same transcription factors are present in both enhancer and the promoter regions (42). Now that we have identified the binding sites in the spacer promoter for rat SL-1 and SF-1, we will be able to determine the relationship between this promoter and the enhancer which lies in the same region (33).

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