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Linker scanner mutagenesis of the *Xenopus laevis* ribosomal gene promoter

Ronald H.Reeder, David Pennock[1], Brian McStay, Judith Roan, Ernie Tolentino and Philippe Walker

Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, WA 98104, USA

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ABSTRACT

We have assayed a series of linker scanner mutants which cover the Xenopus laevis ribosomal gene promoter at approximately ten base pair intervals. All of these mutations adversely affect promoter activity with the exception of one mutation which stimulates activity. Thus, none are neutral. We show that most of the mutations can be partially rescued by ligating a block of enhancer elements upstream of the promoter. In addition, we have made extracts from liver nuclei which produce DNaseI protection footprints over the promoter. Analysis of both strands reveals a prominent footprinting domain from about -5 to -30. However, lesser changes in the digestion pattern are detected over most of the promoter. Previously published analyses have suggested that this promoter might be composed of three functional domains. The experiments presented here suggest that either 1) the three putative domains are so closely arranged that the boundaries are difficult to discern, or 2) the situation is more complex.

INTRODUCTION

Detailed structural analysis of a wide variety of eukaryotic promoters has shown that the large majority are composed of two or more semi-autonomous domains. In general each domain is the binding site for a particular protein (or group of polypeptides) and can often be assigned a particular function (ie, specification of the initiation site, regulation by low molecular weight effectors, tissue specificity, etc.). Interaction between these semi-autonomous domains (and perhaps other non-DNA-binding proteins) combines to yield the fully functional promoter.

In this article we have utilized linker scanner mutagenesis (1) to explore the possible domain structure of the X. laevis ribosomal gene promoter which is recognized by RNA polymerase I. A similar linker scanner study of this same promoter has also been recently reported by Windle and Sollner-Webb (12). Previous studies of this promoter, including deletion mutagenesis (2), DNaseI footprinting (3) and sequence comparisons had led to the provisional conclusion that the promoter is composed of three domains, one of which is an enhancer element (4). However, the linker scanner mutagenesis reported in this paper

has so far failed to detect neutral regions between these putative domains. DNaseI footprinting, an alternate technique for locating promoter domains, does reveal regions which are more protected than others. However, some changes in the digestion pattern are visible over most of the promoter. Thus, delineation of the true domain structure of this promoter must await further work.

METHODS

Construction of linker scanner mutants.

All mutants were made in ψ40, a pBR322 based plasmid which contains a single gene promoter from X. laevis ribosomal DNA linked to a ribosomal minigene body (See Figure 1 for the structure of ψ40 and some relevant restriction sites). Its construction has been described previously (4). In order to distinguish ψ40 transcripts from endogenous ribosomal gene transcripts, ψ40 has 40 bp of linker DNA inserted at position +31. In experiments where an internal standard plasmid was desired we used ψ52 which is identical to ψ40 except that it contains 52 bp of linker DNA at the +31 site.

Enhancer elements were added onto ψ40 by ligating a block of 10 elements (ψ40 + 10E, see Figure 1) or, alternatively, by removing the internal PstI fragments to reduce the number of enhancers to 3 (ψ40 + 3E).

Linker scanner mutations were constructed by inserting an EcoRI site at various places within the promoter region without changing the spacing of the promoter elements on either side of the EcoRI site (1). Insertion of the EcoRI site was done by linking appropriate pairs of matched 5′ and 3′ deletion mutants, each with an EcoRI site at its deletion endpoint. Construction of the 5′ and 3′ deletions was done using exonuclease III and the unidirectional deletion method described by Henikoff (5). For example, to make the 3′ deletions a 295 bp SalI-BamHI fragment from ψ40 was inserted into the polylinker of mp19 (6) and the double stranded form was doubly digested with BamHI and SstI. This leaves a 5′ extended DNA terminus just 3′ to the promoter and a 3′ extended DNA terminus on the other end of the vector sequences. 5μg of this restriction digested DNA was then further incubated with 335 U of Exonuclease III in a total volume of 50μl of 66mM tris-HCl, pH8, 0.66mM MgCl2. Under these conditions exonuclease III digests the 5′ extended terminus at a rate of 40 nucleotides/min but cannot digest the 3′ extend terminus. After an appropriate time of incubation the exonuclease digestion was stopped and the single stranded DNA tails were removed by addition of 150μl of 200mM NaCl, 50mM Na acetate, pH 4.5, 1mM ZnSO4, 0.5% glycerol containing 7.5 U of S1 nuclease and an additional incubation at room temperature for 30 min. The S1 digestion

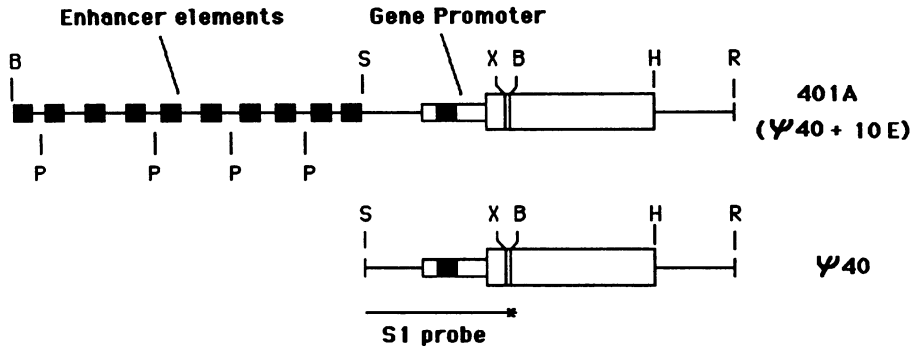

Figure 1. Diagram of ribosomal minigenes used to assay LS mutants.
All LS mutations were placed within the gene promoter present on ψ40, a ribosomal minigene whose construction has been described (4). To test the effect of adding additional enhancer elements a block of 10 elements was added in their normal location upstream of the promoter to yield 401A (ψ40 + 10E).

was stopped by addition of 20µl of 0.5M tris-HCl, pH8, 125mM EDTA and the DNA was extracted with phenol, then with chloroform, and finally precipitated with ethanol. To make certain that all of the DNA termini were flush the DNA was dissolved in 20µl of 20mM tris-HCl, pH8, 7mM MgCl2 and 2.5 units (0.5ul) of the Klenow fragment of DNA polymerase I was added. After incubation at 37° for 15 min the reaction was made 2.5mM in all four dNTP's by addition of 1µl of a stock solution and incubation was continued for a further 15 min. The reaction was then placed at 65° for 10 min to inactivate the DNA polymerase, cooled on ice, and the flush ends were rejoined by addition of 80µl of 50mM tris-HCl, pH7.4, 10mM MgCl2, 10mM dithiothreitol, 1mM spermidine, 1mM ATP, 100ug/ml BSA plus 1µl of DNA ligase for two hours at room temperature. 20µl of the reaction mix was used to transform competent cells. Individual colonies were grown up as single stranded DNA and single base dideoxy sequencing (A-tracks) was done on each to screen for potentially useful deletion endpoints. 5′ deletions were made in the same way as were the 3′ deletions except that the SalI-HindIII fragment of ψ40 was inserted into mp19 and the resultant plasmid was digested with SalI and SstI before the exonuclease III digestion.

As pointed out in reference (7), mp19 has an EcoRI site immediately adjacent to the SstI site. Thus the above procedure automatically results in an EcoRI site being placed at the deletion endpoint without addition of linker oligonucleotides. After matching 5′ and 3′ deletions were identified and ligated together, their structure was verified in each case by checking the size of restriction fragments and by sequencing across the inserted EcoRI site.

To make LS -111/-75, in which most of the enhancer homology region is

replaced by foreign DNA, two complementary oligonucleotides were synthesized, each 28 nucleotides long. When annealed together they formed a double stranded fragment with an EcoRI sticky end at either terminus and the internal sequence shown for LS -111/-75 in Figure 2. LS -111/-102 was digested with EcoRI and SalI and the upstream part of the promoter was isolated. Similarly, LS -83/-75 was digested with EcoRI and BamHI and the downstream part of the promoter was isolated. The upstream and downstream parts were then joined together via the synthetic oligonucleotide in a three part ligation. Subsequent sequencing verified that the spacing between the upstream and the downstream portions was not altered in the final LS -111/-75 mutant.

Assay of mutants by oocyte injection and primer extension.

Oocyte injection and assay of transcription by primer extension was done as described in reference (4). Alternatively, transcription was assayed by S1 nuclease protection as described in reference (8). Oocyte nuclei were injected with about 600pg of a 1:1 mixture of ψ52 (containing a wild type prompoter) and ψ40 (containing various LS mutations in the promoter).

Assay of mutants in an in vitro S-100 extract and assay by S1 nuclease protection.

S-100 extracts of X. laevis cultured kidney cells were made, and in vitro transcription was performed, as described in (8). Transcription was assayed by S1 nuclease protection using a single stranded probe made from the SalI-BamHI fragment of ψ40 and labeled at the BamHI site (described in reference 9, map position illustrated in Figure 1).

DNaseI footprinting with liver nuclear extracts.

Nuclei were isolated from frog livers by the procedure of Gorski, et al. (20) and extracts were prepared as described by Dignam, et al. (21). A double-stranded DNA fragment corresponding to the S1 probe in Figure 1 was labeled at either the SalI or the BamHI site before mixing with extract and digestion with DNaseI as described in reference (22).

RESULTS

Assay of Linker Scanner Mutations

We have constructed a series of linker scanner mutations which cover the Xenopus laevis ribosomal gene promoter in approximately 10 bp intervals. The sequences and locations of these mutations are shown in Figure 2. Each mutation introduces a novel EcoRI restriction site into the promoter sequence and most of them change at least 6 nucleotides of sequence. These mutations have been tested by mixing each one with an equimolar amount of an unmutated promoter and injecting the mixture into the nuclei of homologous X. laevis

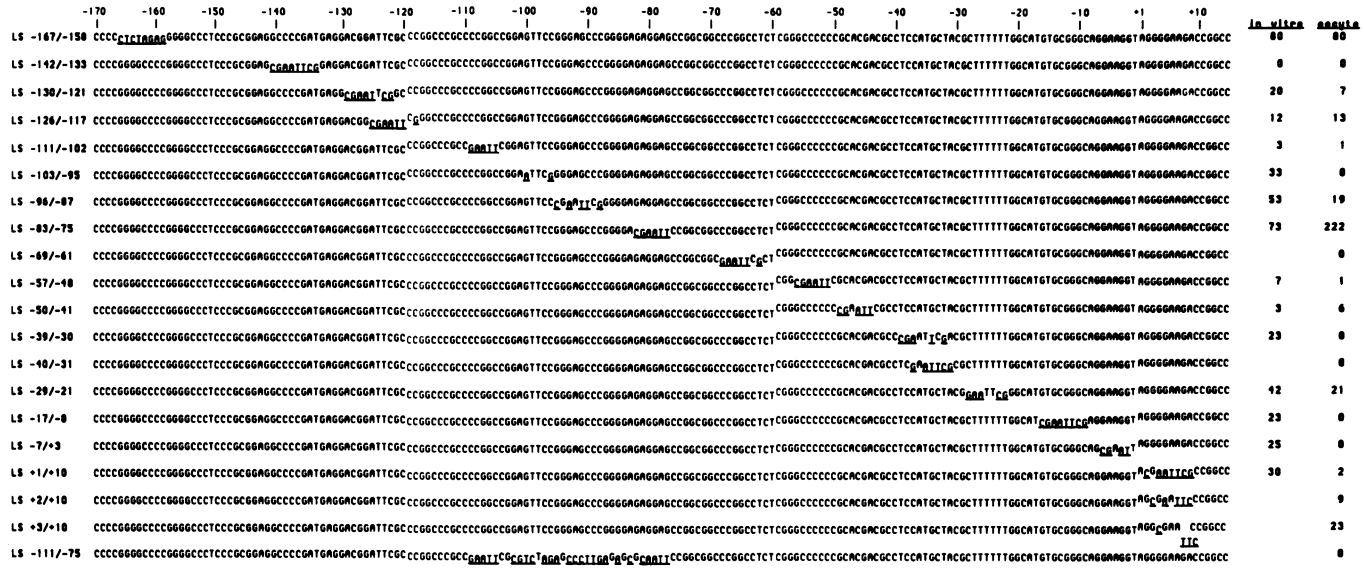

Figure 2. Sequence of linker scanner mutations and summary of transcription assays.

The underlined nucleotides indicate ones which were changed by insertion of the EcoRI linker. Note that except for LS +3/+10 the spacing of the sequences on either side of the inserted mutations is unchanged. The columns on the left indicate the relative transcription activity of each mutant assayed either in vitro or by oocyte injection. The in vitro data are the average of two separate experiments; the oocyte injections of at least two, and most cases six to twelve independent experiments.

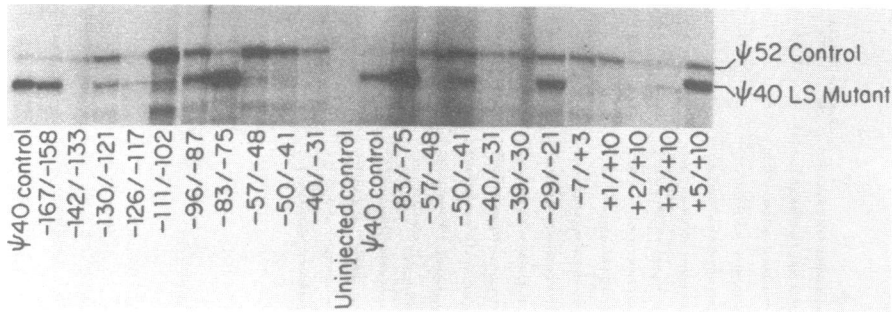

Figure 3. Autoradiograph of typical LS mutant assays.
ψ40 (containing various LS mutations in the promoter) was injected into oocyte nuclei in a 1:1 molar ratio with a control plasmid, ψ52 (containing a wild-type promoter), and transcription was assayed by primer extension. The autoradiograph was traced with a densitometer and transcription signals were normalized to the ψ52 control signal. The results of this and other independent assays are tabulated in Figure 2.

oocytes. After an overnight interval, the amount of steady state RNA initiating from mutant and wild-type promoters was measured by an S1 protection assay using an excess of labeled probe, or by primer extension using an excess of labelled primer. Every mutant was injected on at least two independent occasions; most were assayed from five to eight times. A typical autoradiograph from a primer extension assay is shown in Figure 3, while assays by S1 protection are shown in Figures 5 and 6. Appropriate exposures from each assay were traced with a densitometer and normalized to the signal obtained from the wild-type, internal standard promoter. The averaged result of all injections into X. laevis oocytes are shown in a summary column in Figure 2.

The X. laevis ribosomal gene promoter has been previously determined, by use of successive 5′ and 3′ deletions, to lie within nucleotides -142 to +6 relative to the site of transcription initiation (2,10). The LS mutants are in good agreement with this previous conclusion. The only problem comes at the 3′ boundary where the transcription signal from mutants downstream of +1 was somewhat variable. As we have discussed previously (2), this is probably caused by the fact that the mutation alters the sequence of the transcript itself and may change its stability. For this reason we have not tried to define the 3′ boundary using the LS mutants but have focussed on the internal structure between -142 and +6.

Within the promoter boundaries we have not found any region that is insensitive to mutation. All mutations caused more or less severe damage to the promoter with the exception of LS -83/-75. This mutation consistently

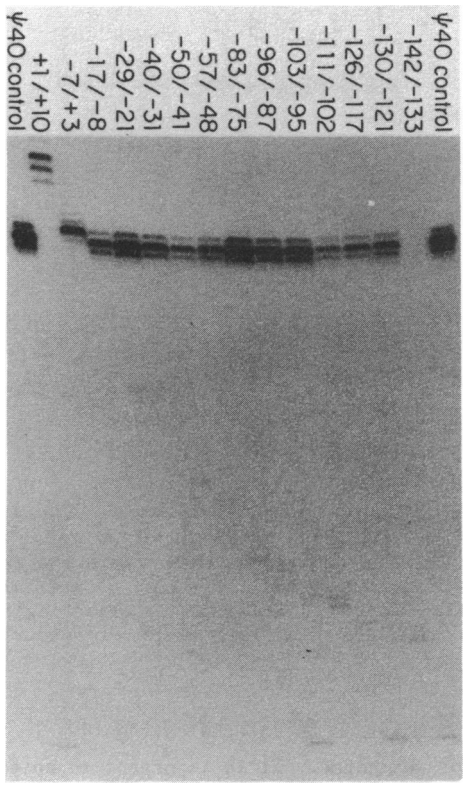

Figure 4. Assay of various LS mutants in vitro in an S-100 extract. Transcription was assayed by S1 nuclease protection and all transcription signals were normalized to the ψ40 control.

stimulated transcription by about two-fold. Thus, this particular set of mutants is unable to detect any neutral regions in the promoter. Either the promoter is all one large domain (rather unlikely, we think) or the domains of the promoter are packed too closely for us to detect their boundaries by this approach.

Most of the LS mutations have also been assayed in vitro with an S-100 transcription extract. An autoradiograph of such an assay is shown in Figure 4 and the quantitation (average of two such experiments) is shown in a summary column in Figure 2. The results are broadly similar to those obtained by oocyte injection. The major difference is that the mutations proximal to the initiation site are less severe in their effect than when assayed by oocyte injection. A possible reason for this difference is that all of the oocyte injections were done with the mutant template in competition with an equal

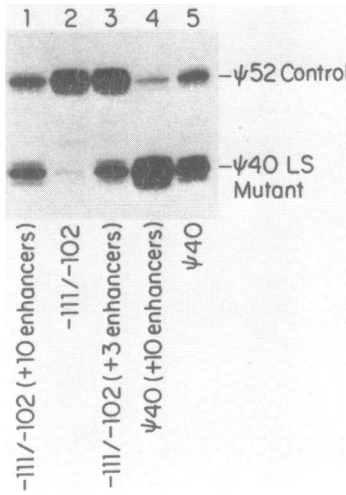

Figure 5. Rescue of LS -111/102 by addition of upstream enhancer elements. Blocks of either 3 or 10 enhancer elements were added onto LS -111/102 (see Figure 1) and each construct was assayed by oocyte injection in the presence of an equimolar amount of ψ52. Note that adding 3 enhancer elements yields a rescue intermediate between the rescue obtained with either 10 or no added enhancers.

amount of wild-type promoter as an internal standard. In the in vitro assays no internal competitor was added. It is important to note, however, that the 5′ boundary of the promoter is the same in both assay systems and all domains of the promoter are required for full activity in the in vitro system.

Rescue of Linker Scanner Mutations by Addition of Enhancer Elements

It has often been noted that the X. laevis ribosomal gene promoter has within it a region that is closely homologous with a domain in each of the enhancer elements that are present further upstream in the intergenic spacer region. This enhancer homology region in the promoter is approximately 42 bp long and extends from position -114 to -72. Within this region LS -111/-102 almost completely abolishes transcription, LS -103/-95 and LS -96/-87 decrease activity about 10-fold and 5-fold, respectively, and LS -83/-75 stimulates transcription 2-fold. These results clearly indicate that the enhancer homology is an important domain of the promoter. We thought it would be interesting to see if mutations within the enhancer homology region could be "rescued" by adding on an additional block of enhancer elements. To test this idea we first attached a block of either 3 or 10 enhancer elements at position -245 upstream of LS -111/-102 (constructs shown in Figure 1). This restores the enhancers to their normal position relative to the gene promoter. As shown

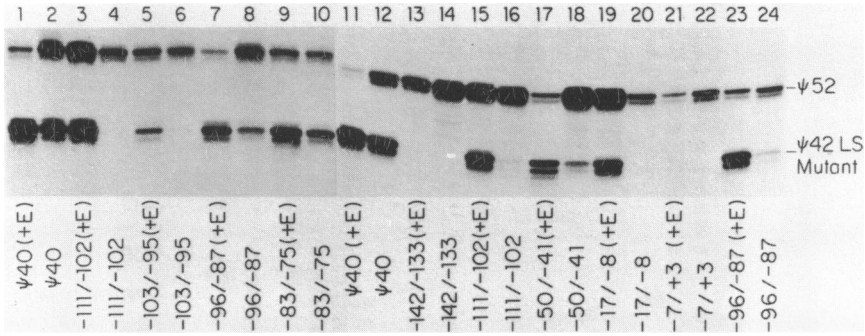

Figure 6. Rescue of various LS mutants by addition of a block of 10 enhancer elements.

Addition of enhancers onto various LS mutants and assay by oocyte injection was the same as in Figure 5. Note that addition of enhancers causes some degree of rescue for all of the LS mutants except LS -142/-133 and LS -7/+3. Close inspection of the original autoradiographs actually showed a slight rescue of LS -7/+3 but we could never detect any rescue of LS -142/-133.

in Figure 5, the LS -111/-102 mutation reduces promoter activity over 10-fold (lane 2). Adding three enhancer elements increases its activity to approximately half of the wild-type level (lane 3) and adding 10 enhancers brings the activity of the mutant up to the same level as the wild type promoter (lane 1). The restoration of activity is roughly proportional to the number of enhancers added. However, even adding 10 enhancers onto the mutant promoter does not yield the same activity as is seen when 10 enhancers are added onto the wild type promoter (lane 4).

When we planned these enhancer rescue experiments, we thought it possible that only mutations within the enhancer homology would be rescued by adding on an enhancer block. In fact, we found that every LS mutant except LS -142/-133 was rescued to some extent (examples of rescue experiments are shown in Figure 6). The degree of rescue corresponded fairly well with the severity of the original LS mutant. In other words, addition of an enhancer block to a severe mutant like LS -111/-102 brought it back up to the level of a wild-type promoter (Figure 5, lane 1; Figure 6, lanes 3 and 13). Addition of enhancers to a less severe mutant such as LS -96/-87 restored it to a level higher than wild type (Figure 6, lanes 7 and 23). Our overall conclusion is that most of the LS mutants weakened the promoter to varying degrees but did not completely inactivate it. Addition of enhancers caused a stimulation (or rescue) of the weakened promoter, the final activity depending upon just how deleterious the original LS mutation was. The only exception to this general rule was LS

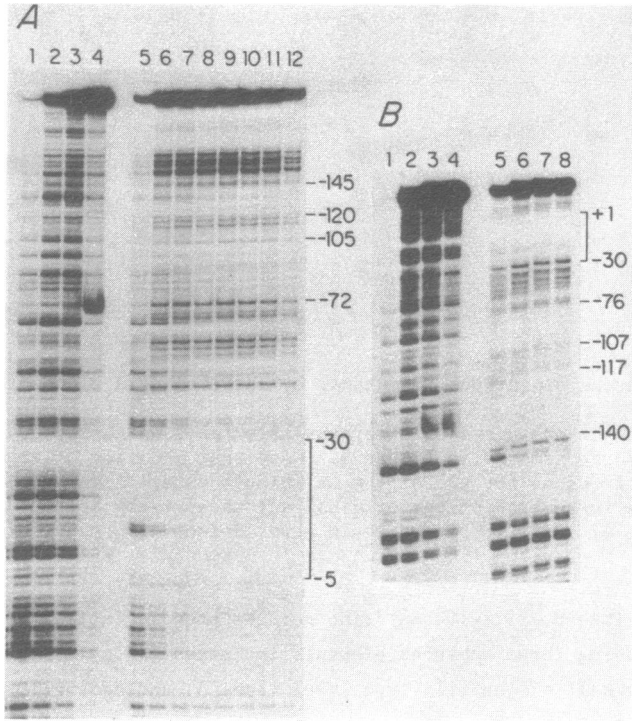

Figure 7. DNaseI footprints on the ribosomal gene promoter.

Panel A. A 300 bp fragment (the same sequence shown as the S1 probe in Figure 1) was labeled at the BamHI site and used for DNaseI footprinting as described in reference (22). Lanes 1-4, naked DNA, varying DNaseI amounts (the heavy band in lane 4 at position -72 is an artifact unrelated to the presence of protein extracts); lanes 5-12, extract plus DNA, increasing DNaseI amounts. The nucleotide numbers were determined from sequencing ladders run in parallel (not shown) and refer to distances from the initiation site at +1. Marked protection is visible in the region from about +1 to -30. Note, however, that changes in the digestion pattern can be seen throughout the rest of the promoter as well.

Panel B. Same as Panel A except that the 300 bp fragment was labeled on the opposite end at the SalI site. Lanes 1-4, naked DNA; lanes 5-8, extract plus DNA.

-142/-133. We could detect no enhancer rescue of this mutant upon repeated assay. It is also true that this mutant was completely dead in every assay we tried. Perhaps it is a true null mutant and no amount of enhancers can rescue it. (LS -7/+3 appears not to be rescued in Figure 6, lanes 20 and 21, but it showed a slight but reproducible rescue on the original autoradiograph).

Complete Replacement of the Enhancer Homology and Attempted Rescue

As shown in Figure 2, we have also constructed a mutant in which the

entire enhancer homology region has been replaced by foreign sequence (LS -111/-75). This construct showed no activity when assayed by itself and was not rescued to any detectable extent by addition of a block of enhancers upstream (data not shown).

Correlation of Linker Scanner Mutations with DNAseI Footprints

Previous DNAseI footprints of the gene promoter suggest that the promoter can be divided into three domains, labeled domains I, II, and III (3). The original footprints were obtained under somewhat difficult conditions. They required predigestion of the immature oocyte extracts with micrococcal nuclease and we were only able to obtain footprints on one strand of the DNA. More recently we have been able to obtain extracts from frog liver nuclei which yield DNaseI footprints on both strands, as shown in Figure 7. The footprints in Figure 7 reveal a major protected region which extends approximately from -5 to -30. (The -30 boundary is difficult to establish precisely since DNase tends not to cut in this region in both the control and the experimental samples). This region overlaps with what we previously called Domain I. Upstream of this fairly clear footprint there are a number of changes in the digestion pattern on both strands (both protections and enhancements) extending at least to the 5′ boundary of the promoter at -142. However, the protections are less pronounced than in the -5 to -30 region and it is difficult to arrange them into clear domains separated by neutral regions. There is clearly much information here which awaits further analysis. But at this point the general conclusion from the footprinting is similar to the conclusion from the LS mutations: proteins change the digestion pattern of most of this promoter and clearly defined domains cannot be unambiguously discerned by this method.

DISCUSSION

The clear implication of this study is that there are few, if any, empty spaces in the X. laevis ribosomal gene promoter. Scanning it at a resolution of about 10 bp shows that every mutation either severely damages the promoter or, in one case, stimulates it. The DNaseI footprinting is consistent with this conclusion. In addition to the prominent protection over the -5 to -30 region there are a series of lesser, but reproducible protections and enhancements over the rest of the promoter with little reason to group them in discrete domains. Indirect evidence still suggests that different parts of this promoter fulfill different functions and that the promoter will ultimately be revealed as a set of interacting domains. For example, under conditions where very high amounts of template are injected, the region downstream of -7 is sufficient for accurate initiation (2,11). And the region

from -72 to -114 is probably an internal copy of an enhancer element. However, the precise delineation of these and other domains must probably await isolation of the protein factors which interact with them.

Windle and Sollner-Webb have published two recent studies relating to the domain structure of the X. laevis ribosomal gene promoter (11,12) and it is instructive to compare their results with ours. We agree on the overall boundaries of the promoter (about -142 to +6). However, our respective assay procedures differ in their degree of dependence on the upstream regions of the promoter. By injecting large amounts of template into the oocyte they are able to obtain accurate initiation with 5′ deletions extending down to -7 (11). This observation is the major reason for concluding that Xenopus has a "core promoter" similar to the core promoter which has been detected in Acanthamoeba (17), Drosophila (13) and mammalian ribosomal genes (18,19). In our hands the entire Xenopus promoter is required for activity even at high DNA inputs and the "core promoter" is completely dead by itself. Likewise, in a recent linker scanner analysis of the X. laevis gene promoter (mutations assayed by injection of lower DNA amounts so that the requirement for the upstream regions could be detected), Windle and Sollner-Webb found that mutation of the enhancer homology region had relatively little effect on transcription (12). In fact, replacement of the entire homology region with foreign DNA had almost no effect. In our hands, mutation of the enhancer homology either decreased or stimulated transcription and replacement of the region killed the promoter completely. We have discussed these results with the Sollner-Webb group several times without finding a reason for the difference in our assay systems. Our common conclusion is that these results emphasize the caution that must be exercised in relying exclusively on any single set of experiments.

RNA polymerase I promoters from a number of other species have been studied, and, in the case of the human polymerase I promoter, linker scanner mutants have been constructed and analysed (7). Aside from some broad generalities, however, it is not yet possible to say whether or not they share a common structure. In general, polymerase I promoters from Drosophila (13), Xenopus (2,10), mouse (14), and human (15,16) have all been shown to overlap the region they transcribe by a few nucleotides and to be of roughly similar size. In addition, under relaxed conditions the region near the initiation site can direct specific initiation by itself and thus can be considered as a "core" promoter. There is not enough data to say whether they have the same types of internal domains or whether they are similarly arranged. In fact, it has yet to be demonstrated that any polymerase I promoter other than the Xenopus promoter utilizes enhancers.

[1]Present address: Department of Biology, University of Rochester, Rochester, NY 14627, USA

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