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Xenopus ribosomal gene enhancers function when inserted inside the gene they enhance

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ABSTRACT

The ribosomal DNA of Xenopus laevis contains repeated sequence elements in the intergenic spacer region that enhance transcription from the adjacent gene promoter (1,2). Previous work has shown that these RNA polymerase I enhancers influence the target promoter when they are in either orientation, at a distance of several kilobases, and only when they are in cis (3-5). In this work, we further show that enhancer activity is unaffected by inserting the enhancers within the transcription unit whose promoter is being enhanced. In addition, enhancer activity does not interfere with transcription through its sequences. The results suggest that the enhancers act at a point prior to the initiation of transcription and that they are likely to be dispensable once transcription has begun.

INTRODUCTION

Enhancers are DNA segments which augment transcription from promoters located in cis. They have been operationally distinguished from elements of promoters by the fact that enhancers are orientation independent and can exert their influence over distances up to several kilobases from the target promoter (6). The first enhancers to be discovered were located in viral genomes and were specific for promoters transcribed by RNA polymerase II (7,8). Subsequently, they have been found associated with a variety of non-viral promoters including promoters for genes that are developmentally regulated (9-11). In addition, we have reported that sequences in the intergenic spacer region of the Xenopus laevis ribosomal genes have enhancer-like activity on the RNA polymerase I promoter that directs transcription of the 7.5 kilobase rRNA precursor (4). The observation that promoters for RNA polymerase I as well as for RNA polymerase II utilize enhancers strengthens the idea that

enhancers perform a vital function that may turn out to be required by all eukaryotic promoters.

The molecular mechanism by which enhancers function is still a matter of some debate. Nor is it established that all enhancer-like sequences even use the same mechanism. For RNA polymerase II enhancers, data is beginning to accumulate suggesting that they are binding sites for some specific protein (12,13). Whether this protein is the RNA polymerase itself, some transcription factor whose eventual role is to translocate to the promoter, or a protein whose role is to stay at the enhancer and help establish "active" chromatin structure is still unclear. In the case of the Xenopus ribosomal gene enhancers, the fact that they can compete against the promoter itself (and that they share sequence homology with a domain in the promoter) suggests that they may serve as attraction sites for one of the auxillary transcription factors needed to establish the stable promoter complex. This model would then imply that enhancer action is only required to set up the active transcription complex and once transcription begins, the enhancers are dispensable. Alternatively, one might envision models in which enhancer function is continuously required (if, for example, they were entry sites for RNA polymerase).

In this paper, we report experiments which show that the action of Xenopus ribosomal gene enhancers is unaffected by inserting them immediately downstream of their target promoter within the coding region even though transcription through the enhancers proceeds uninhibited. This result is consistent with a model in which the enhancer's only function is to attract a transcription factor and help to establish transcriptional activity. Once this is accomplished, the enhancers may be dispensable.

EXPERIMENTAL PROCEDURES

Oocyte injections were performed as described previously (3,4). All plasmids were injected in the presence of 500ug/ml α-Amanitin. For S1 analysis, RNA was hybridized to a single-stranded ^{32}P-end-labelled probe in 0.3M NaCl, 10mM Tris-HCl (pH 7.6), 1mM EDTA at 80^{o}, and the hybrids were treated with 45 units

of S1 nuclease in 50mM NaCl, 30mM NaAcetate (pH 4.5), 1mM $ZnSO_4$, 5% glycerol for 30 minutes at 37°C. DNA fragments protected from S1 nuclease were run on 8% denaturing polyacrylamide gels.

RESULTS

The Xenopus laevis ribosomal gene enhancers occur as clusters of repetitive elements that are either 60 or 81bp in length (14). Most of our experiments have been done with a block of ten of these 60/81bp repeats which we employ as an enhancer cassette.

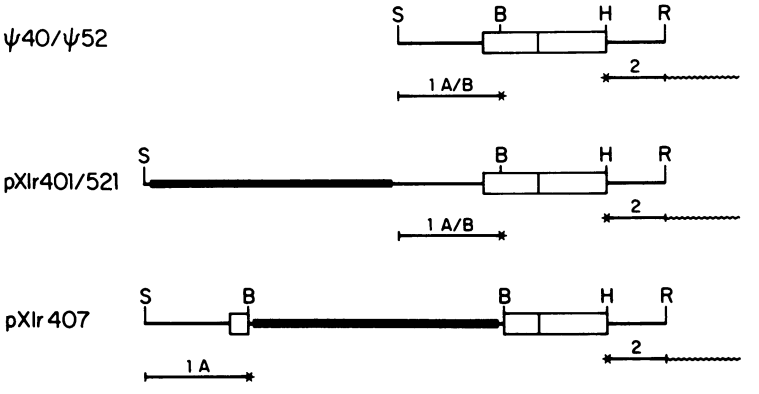

Figure 1. Structure of ribosomal gene plasmids and probes used to assay their transcription. ψ40, ψ52, pXlr401, and pXlr521 have been described previously (4). In short, ψ40 and ψ52 contain 245bp upstream from the 5' end of the 40S coding sequence (including the promoter) and the first 115bp of the 40S coding sequence. These are directly linked to 200bp from the 3' end of the 40S (28S) coding sequence plus 160bp adjacent spacer sequences. pXlr401 and pXlr521 are the same as ψ40 and ψ52 except having 980bp upstream sequences, thus including the enhancer. ψ40 and pXlr401 contain 40bp of linker DNA inserted at position +31 (including the BamHI site), ψ52 and pXlr521 have 52bp inserted. In pXlr407, the fragment containing the enhancer was moved from its natural position into the BamHI site of pXlr401. Probes: Probe 1 (SalI-BamHI fragment) was made from ψ40 (1A) to detect transcripts from ψ40, pXlr401, and pXlr407, and from ψ52 (1B) to detect transcripts from ψ52 and pXlr521. Correct transcripts protect 50 and 62 nucleotides, respectively, from S1 digestion. Probe 2 is a 3' end labelled HindIII-PvuII fragment of a corresponding pSP64 clone, and therefore its sequence diverges from the sequence of the template (a pBR322 clone) past the EcoRI site (wavy line). Read-through RNA from the HindIII to the EcoRI site protects 165 nucleotides of the probe from S1 digestion. Restriction sites: S, SalI; B, BamHI; H, HindIII; R, EcoRI. Black bar, enhancer; open boxes, 40S precursor coding regions.

The plasmid constructions used in this work are shown in Figure 1. The standard assay for enhancer activity is a competition assay in which two ribosomal minigenes on separate plasmids are co-injected in equimolar amounts into the same oocyte (3). The RNA is analyzed with a pair of S1 probes by which the transcripts from the two co-injected plasmids can be detected (Fig. 1, probes 1A and 1B). If neither of the minigene constructs carries an enhancer cassette, then equal transcription is observed from each (compare the equal signals in Fig. 2 from ψ40, lane 1, and ψ52, lane 2). In contrast, if one of the minigenes has an enhancer cassette in the normal position upstream of the promoter, then it will give a signal 10 to 20-fold greater than the signal of the enhancer-less partner (compare the large signal in Fig. 2 from pXlr521, lane 4, with the smaller signal from ψ40, lane 3).

In the constructs pXlr401 and pXlr521 the enhancer cassette is located in its normal location and orientation upstream of the gene promoter. To test the effect of placing the enhancers within the transcription unit we constructed pXlr407 (see Fig. 1) and tested its activity by oocyte injection. When pXlr407 is co-injected with ψ 52 (which has no enhancers), pXlr407 is strongly dominant indicating that the enhancers are unimpaired by inserting them within the genes (Fig. 2, lanes 7 and 8). This conclusion is supported by coinjecting pXlr407 with a construct that has enhancers in the normal position (pXlr521). As shown in Figure 2, lanes 5 and 6, both plasmids give equal signals in this case. Since enhancers only act in cis, we can exclude the possibility that enhancer action and transcription occur on different individual plasmids. We conclude that transcription into the enhancer sequence does not impair its activity.

In the experiments shown in Figure 2, transcription was assayed by using S1 nuclease protection to measure the formation of correct 5' termini. This assay measures the steady state amount of only the first 50 or 62 nucleotides of the various transcripts. The question arises, therefore, whether or not polymerase actually transcribes through the enhancer sequence in pXlr407. To examine this point, we measured transcription in a region downstream from the enhancer insert in pXlr407 and compared its level to the transcription in the same region in

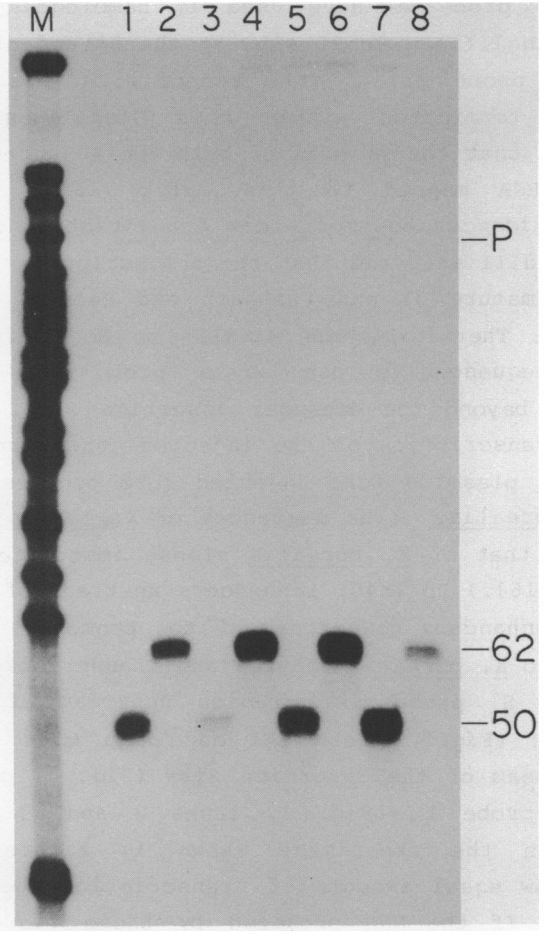

Figure 2. Competition assay for enhancer activity. Equimolar amounts of the following pairs of constructs were injected into oocytes of X. laevis: Lane 1 and 2, ψ40 and ψ52; lane 3 and 4, ψ40 and pXlr52l; lane 5 and 6, pXlr407 and pXlr52l; lane 7 and 8, pXlr407 and ψ52. From each injection, one aliquot of RNA was analyzed using a ψ40-specific S1 probe (probe 1A, lanes 1,3,5,7) and another aliquot was analyzed using a ψ52-specific S1 probe (probe 1B, lanes 2,4,6,8). Virtually no full-length protection of the probe indicating read-through transcription is detected (P). Note that the similar signals in lanes 1 and 2 provide a test for the similar specific activity of the probes 1A and 1B, since similar signals are to be expected with this pair of constructs from earlier studies using a primer extension assay (4). M, end-labelled HpaII-digest of pBR322.

pXlr401. The probe we have used is homologous to sequences between the HindIII and EcoRI site at the 3' end of the minigene (see Fig. 1, probe 2). Until recently it was thought that transcription terminated within this HindIII site since S1 mapping showed that the 3' ends of both the 40S precursor and the mature 28S rRNA mapped to this point (15). We now have unequivocal evidence, however, that transcription continues well beyond the HindIII site and that the transcript is then processed to yield the mature 3' end (Labhart and Reeder, manuscript in preparation). Therefore, the HindIII to EcoRI fragment is an appropriate sequence to use as a probe for transcription continuing on beyond the enhancer insertion site. In order to distinguish transcription of the injected genes from endogeneous transcription, plasmids were injected into oocytes of a related species, X. borealis. (The sequences of X. laevis ribosomal DNA diverges from that of X. borealis almost immediately beyond the HindIII site [16].) pXlr401 (enhancers upstream of the promoter) and pXlr407 (enhancers downstream of the promoter) were injected separately into X. borealis oocytes and transcription was assayed using both an S1 probe to a region upstream of the enhancer insertion site (Fig.1, probe 1A) and also with a probe to a region downstream of the insertion site (Fig. 1, probe 2). The results with probe 1A (Fig. 3, lanes 2 and 3) show that in agreement with the experiment shown in Figure 2, the two constructs show equal amounts of transcription when the 5' ends are assayed. If the RNA produced by those two constructs is assayed with probe 2, the downstream region is found to show equal amounts of transcription as well (Fig. 3, lanes 5 and 6). Lane 4 shows that the transcription we detect past the HindIII site is truly promoter dependent (and not due to some non-specific background transcription) since a plasmid similar to pXlr401 that has a promoter deletion (see lane 1) but contains an enhancer shows no transcription in this region. We can conclude from this result that no polymerases terminate within the enhancer region in pXlr407. Note that it is not relevant for this argument to know the relative specific activities of the probes 1A and 2 or to demonstrate that the downstream RNA is as abundant as the promoter RNA, since we are only interested in

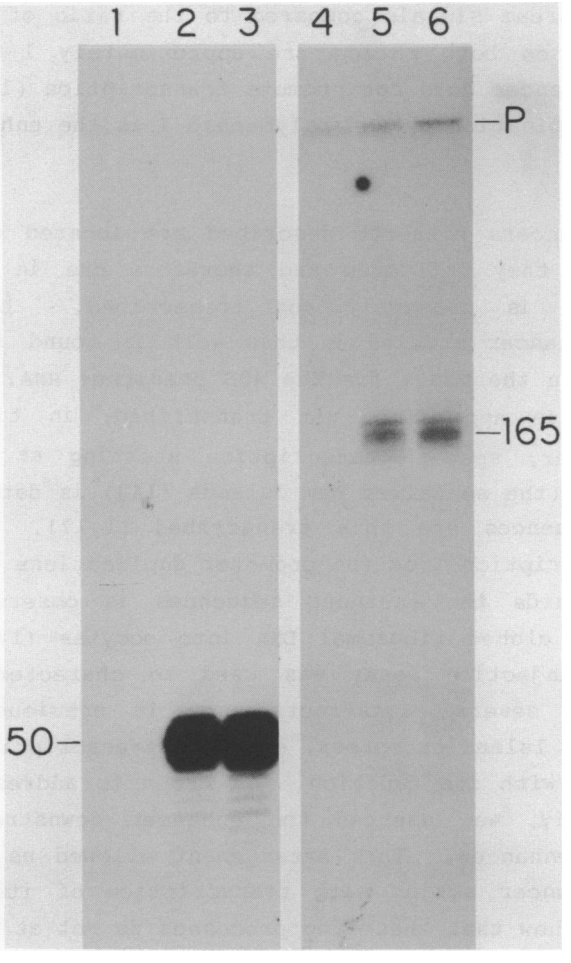

Figure 3. Assay for the efficiency of transcription through the enhancer. pXlr401 (lane 2 and 5) and pXlr407 (lane 3 and 6) were separately injected into oocytes of X. borealis and the RNA of the injected oocytes was analyzed by S1 protection assay using either probe 1A (lane 2 and 3) to detect 5' termini of the 40S precursor, or probe 2 (lane 5 and 6) to detect transcription past the HindIII site. As a control, a construct similar to pXlr401, but having the promoter deleted, was injected and likewise analyzed using the two probes 1A (lane 1) and 2 (lane 4).

comparing the ratios of promoter-transcription in pXlr401: promoter-transcription in pXlr407 and downstream-transcription in pXlr401:downstream-transcription in pXlr407. Any termination events within the enhancer would have to lead to a higher ratio

of the downstream signals compared to the ratio of the promoter signals. Since both ratios are approximately 1 (Fig. 3) and since the enhancer does not promote transcription (lane 4), there can be no termination by RNA polymerase I in the enhancer region.

DISCUSSION

Most enhancers hitherto described are located upstream from the promoter they influence and therefore are in a non-coding region which is presumbly not transcribed. Likewise, the ribosomal enhancer studied in this work is found in the spacer region between the genes for the 40S precursor RNA. In oocytes, this spacer is apparently not transcribed; in tissue culture cells, however, spacer transcription starting at the promoter duplications (the so-called Bam Islands [14]) is detected and the enhancer sequences are thus transcribed (1,17). Furthermore, spacer transcription from the promoter duplications in the spacer directed towards the enhancer sequences is observed following injection of cloned ribosomal DNA into oocytes (17,18). Since the oocyte injection assay was used to characterize enhancer function and several constructs used in previous work (1-3) contained Bam Island promoters, enhancer transcription seemed not to interfere with its function. In order to address this point more carefully, we inserted the enhancer downstream from the promoter it enhances. This arrangement allowed us to correlate directly enhancer action with transcription of its sequences. The results show that these two processes do not at all interfere with each other.

Electron micrographs of active ribosomal genes show that polymerase density can be as high as one polymerase per 60-75 basepairs which is likely to be as close as these large protein assemblages can be packed on a DNA chain (see e.g. 18). The same polymerase density can also be found on injected cloned ribosomal genes (18-20), and since transcription signals obtained from plasmids containing a full ribosomal gene repeat (as pXlrl01A, 20) and from minigene constructs as the ones used in the present study are of similar intensity (2), the polymerase density on minigene-constructs like pXlr401 and pXlr407 has to be comparable to the one on full-repeat clones. Clearly, we cannot be certain

that all injected plasmids that are active are transcribed at such a maximal rate. Still, it is a reasonable assumption that a considerable proportion of the transcription observed from injected plasmids derives from genes bearing a high polymerase density. The results therefore suggest that the enhancer block functions just as well when it is traversed by these close-packed polymerases as it does when located upstream of the promoter. This observation alone would seem to rule out the possibility that the enhancers serve as (bidirectional) polymerase entry sites since it is difficult to envision how an entering polymerase could then swim upstream against the polymerases already chain elongating to reach the initiation site. The same argument can also be made in the cases where polymerase II enhancers have been identified inside the transcription unit. However, the argument is much stronger for the ribosomal gene case since electron microscopy gives us an independent estimate of polymerase packing density. If the polymerases are more sparsely packed (as is often the case for polymerase II genes), then it is just possible that each polymerase could enter at an enhancer within the gene, move upstream to the initiation site, and then elongate past the enhancer before the next polymerase entered and thus avoid any collisions. The available data suggest that such a scenario is impossible for the ribosomal gene enhancers. We are aware of the formal possibility that the polymerase could enter at an enhancer within the gene and move in the direction of transcription around the circular plasmid to the promoter. However, for such a mechanism not to interfere with transcription, the moving (sliding?) polymerases would have to travel at the same speed as the transcribing polymerases. No data supporting such a model are available.

A second model of enhancer action proposes that some protein binds to the enhancer and stays there to exert its action. (Such a protein could, for example, be a site of attachment to the chromosome scaffold or nuclear matrix.) Again,it seems unlikely that such permanent binding and action could be tolerated in a region through which close-packed polymerases were continually elongating. Nor does it seem likely that such a bound protein would have no effect on the transcription rate through this

region. However, it is clear that in some cases polymerase can transcribe through a bound protein (polymerase III transcribing through transcription factor IIIA on the 5S genes is an example [21]). Although we think it is highly unlikely, it is not possible to completely rule out such a model at present.

Considering all the data available about the Xenopus ribosomal gene enhancers, we think it is most likely that they are attraction sites for a transcription factor whose eventual role might be, for example, to translocate to the gene promoter and help establish the stable, open promoter complex. In such a model, enhancers are needed only at the beginning to establish transcriptional activity. Once this is achieved, polymerase could proceed through the enhancers with impunity.

We should also point out that there is a similarity between our construct pXlr407 and the structure of immunoglobulin genes where an active enhancer is likewise located in the coding region (9,10). Results with the immunoglobulin enhancer also suggest that the enhancer is involved in establishing transcription but not in its maintenance since heavy-chain gene expression has been found to continue at high levels even after deletion of the enhancer region (22,23).

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