Variations in transcriptional activity of rDNA spacer promoters

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ABSTRACT

We have compared the DNA sequences of several different examples of the duplicated polymerase I promoters that are found in rDNA spacers of *Xenopus laevis*. Although different spacers exhibit different amounts of transcription *in vivo*, this does not seem to be due to DNA sequence differences between spacer promoters. We have found that several different spacer promoters when subcloned and injected into oocytes exhibit similar promoter activities when transcription is assayed by primer extension analysis. Moreover, the activity of these spacer promoters is the same as that of a co-injected gene promoter. The equivalence of spacer promoter activity and gene promoter activity was also found when rDNA plasmids containing intact spacers were injected into oocytes and transcription assayed by primer extension. This is in contrast to (1) the inactivity normally exhibited by the promoters of endogenous spacers in oocytes, (2) the relative inactivity of spacer promoters found when transcription of the same rDNA plasmids is assayed by electron microscopy.

INTRODUCTION

The spacers that separate the ribosomal RNA genes of *Xenopus laevis* contain two or more imperfect duplications of the entire RNA polymerase I promoter (1-3). Both electron microscope and biochemical studies of endogenous oocyte ribosomal DNA (rDNA) have shown that usually these spacer promoters are either silent or far less active than the gene promoter (4-6). However, in the oocytes of rare individuals many of the endogenous spacer promoters are active (7,8). We would like to know the molecular basis for this range of spacer promoter activities. One simple explanation could be that transcriptionally-active spacer promoters are more similar in DNA sequence to the gene promoter than are the promoters of inactive spacers. We show here that this explanation is unlikely.

In our attempts to correlate the activity of various promoters injected into oocytes with the DNA sequence of these promoters, we have found that both having spacer DNA on a microinjected plasmid and the method used to assay transcription affect the amount of activity exhibited by spacer promoters.

MATERIALS AND METHODS

Structure of ribosomal DNA plasmids

See Fig. 1 for diagrams of rDNA inserts. pXlr164 and pXlr101A are full-length repeat units of ribosomal DNA (gene plus spacer) that have been cloned from separate frogs (8,10). pXlr264 is a HinfI fragment that was subcloned from the spacer of pXlr164 (8). pXlr211 was derived from the parent plasmid pXlr14 (12) by removing most of the gene region and fusing the residual 5' and 3' terminal fragments together to create a 315 bp minigene (13). pXlr211 also has 160 bp of 3'-proximal spacer left intact. pXlr409 is a derivative of pXlr211 from which the central three spacer promoters and associated sequences have been deleted to create a shorter spacer with only two promoters. In addition, the minigene body of pXlr409 has an extra 40 bp of linker DNA inserted at the +31 position so that its transcripts can be distinguished from endogenous rRNA transcripts by primer extension analysis. ψ 40 is the same as pXlr409 except that the spacer upstream of -245 has been removed. ψ 52 is the same as ψ 40 except that it has a 52 bp insert at position +31. pXlr164G was made by adding SalI and BglII linkers to a 300 bp SmaI fragment from pXlr164 that contains a spacer promoter (underlined in Fig. 1) and ligating it in the desired orientation to a Bam HI site in the 40 bp linker insert of ψ 40. pXlr101G was made in a similar fashion using a 300 bp SmaI fragment from the spacer of pXlr101A; pXlr14G was made using a spacer fragment from pXlr409. Transcripts from pXlr164G, pXlr101G and pXlr14G can all be assayed by primer extension using the same primer as is used to detect transcription from the gene promoters of 164, 101A, 211, 409, ψ 40 and ψ 52. All constructs were injected as inserts in circular pBR322, and in solutions containing 500 μ g per ml of α -amanitin.

Assays of promoter activity

Transcription from rDNA promoters was measured using primer extension analysis as described previously (13). The primer used to detect transcription from all gene promoters and from spacer promoters in minigene constructs was an 88 nucleotide fragment from a ClaI site at +116 to a HaeII site at +28 relative to the gene initiation site. Transcripts from endogenous genes give a signal that is 116 nucleotides long, the ψ 40 signal is 156 nucleotides, the spacer promoter(s) signals are 162 nucleotides and the ψ 52 signal is 168 nucleotides. The primer used to detect transcription from spacer promoters in intact spacers was a 31 bp fragment from an AvaI site at +16 to an AvaII site at +47 relative to the site of initiation in the spacer. Both primers were 5' end-labelled by polynucleotide kinase to approximately the same specific activity.

Deletion analysis has shown that sequences between -142 and +6 are normally required for promoter function (15,16). Under some experimental conditions we have found that a small domain surrounding the initiation site is sufficient for accurate initiation (16). However, under most experimental conditions (for example, competition) this is not the case (15,16, unpublished observations). In the experiments reported here conditions were such that the full -142 to +6 promoter region was utilised.

RESULTS

Sequence of spacer promoters

In order to determine whether active spacer promoters are notably different in DNA sequence from promoters of other spacers we compared the sequences of promoters from several different spacers. We have previously reported the cloning of a repeat unit of rDNA (pXlr164) from an individual whose spacers showed a high level of transcription in electron microscope preparations of oocyte rDNA (8). The DNA sequence of the spacer promoter farthest from the gene in pXlr164 (underlined in Fig. 1) was determined and is shown in Fig. 2. For comparison we also sequenced a spacer promoter from

Fig. 1 Structure of ribosomal DNA plasmids.

The thick bars represent the 5' (cross-hatched) and 3' (plain) transcribed regions of the rRNA gene. The black boxes represent 42 bp repeated elements that have enhancer-like properties (14) and that have close sequence homology with an internal region of both gene and spacer promoters. Details of the cloning and construction of the various inserts are given in Materials and Methods.

Fig. 2 Sequences of various *X. laevis* spacer promoters compared to the gene promoter. The sequence of the gene promoter (2,3) is shown at bottom numbered relative to the site of transcription initiation. Xlo8(a) and (b) are two spacer promoters from the spacer sequenced by Boseley, et al and Moss and Birnstiel (1,2). 14(e) is from the independent clone originally sequenced by Sollner-Webb and Reeder (3) and is the promoter present in pXlr14G (Fig. 1). 101(a) is from pXlr101A (10) and is the promoter present in pXlr101G (Fig. 1). 164(a) is from pXlr164 (8) and is the promoter present in pXlr164G (Fig. 1). We determined the sequence of 101(a) and 164(a) using dideoxy sequencing of inserts in M13 mp10 and mp11. Triangles above the line indicate insertions; triangles below the line indicate deletions. Dashed lines indicate identity with the gene promoter sequence.

pXlr101A, a clone we have used in numerous other oocyte injection experiments (10). Also included in Fig. 2 are the sequences of three other spacer promoters that have been previously reported. Two facts emerge from this comparison. (1) Although none of the promoters are identical, all of them are very similar to each other and all differ from the gene promoter at 14-15 positions out of the 149 bp (-145 to +4) that constitute the promoter duplication. (2) The spacer promoters differ from each other at only 1 or 2 positions. The spacer promoter from pXlr164, which we know was active in its endogenous location, does not exhibit a unique sequence change with respect to other promoters. Our conclusion from the data in Fig. 2 is that sequence differences in the promoters themselves are probably not responsible for differences in endogenous spacer activity.

Transcriptional activity of isolated spacer promoters

The above conclusion was strongly reinforced when we measured the activity of individual spacer promoters by subcloning them and re-injecting them into oocyte nuclei. A 300 bp SmaI fragment containing a spacer promoter (underlined in Fig. 1) was removed from three different spacers and attached to the body of the rDNA minigene Ψ 40 to create the new minigenes pXlr 164G, 101G, and 14G. Each of these new minigenes was mixed with an equimolar amount of either Ψ 40 or Ψ 52 (minigenes that contain the unaltered gene promoter) and injected into oocyte nuclei. Since all of these minigenes shared the same gene body, transcription from all of them could be assayed by primer extension using the same primer. The result, shown in Fig. 3 was that all three subcloned spacer promoters had the same promoter strength and this promoter strength was essentially the same as that of the gene promoter. So despite the fact that spacer promoters differ from the gene promoter at 14 or 15 positions, the transcriptional activities of spacer and gene promoters were equivalent by this assay.

Transcriptional activity of promoters in intact spacers

It might be argued that the spacer promoters all appear active in Fig. 3 because in the process of subcloning them we have removed them from some inhibitory sequence elsewhere in the spacer. Therefore, in the experiment shown in Fig. 4 we injected into oocytes various rDNA clones that contained genes and intact spacers and used two different primer extension probes to assay simultaneously the activity of both spacer and gene promoters. Although the gene promoter activity was somewhat variable in this experiment, the spacer promoters were uniformly active and produced transcripts at a rate as high or higher than the gene promoters. These observations agree with a previous report from Moss (9) who also found active spacer transcription on

injected plasmids.

We have also used the same two probes to compare transcription from the endogenous spacer and gene promoters of several representative cell types. Oocytes usually showed little or no detectable spacer transcription (Fig. 5a, lanes 1 and 3; Fig. 5b, lanes 3 and 6); in embryos a small amount of spacer transcription was usually detectable (Fig. 5b, lanes 1 and 4). It should be noted that at this developmental stage at least part of the gene promoter signal is due to stored precursor RNA left over from oogenesis(11). However,

Fig. 3 Comparison of transcription from subcloned spacer promoters and gene promoters after injection into oocytes.

Spacer promoters from pXlr164, pXlr101A and pXlr409 were fused to a promoterless-minigene to form 164G, 101G and 14G as shown in Fig. 1. Equimolar amounts of each fusion gene were injected into *X. laevis* oocyte nuclei either singly or in combination with equimolar amounts of $\psi 40$ or $\psi 52$, plasmids which carry a gene promoter and serve as internal controls and competitors for the spacer promoters. Transcription from each promoter was assayed by primer extension of total RNA extracted from 5 oocytes using the same single-stranded primer for all of the injected genes. Note that $\psi 52$ reproducibly gives a slightly reduced signal compared to other gene or spacer constructs(14).

The injected plasmids were: 101G, lane 1; 101G plus $\psi 40$, lane 2; 101G plus $\psi 52$, lane 3; 164G, lane 4; 164G plus $\psi 40$, lane 5; 164G plus $\psi 52$, lane 6; 14G, lane 7; 14G plus $\psi 40$, lane 8; 14G plus $\psi 52$, lane 9; $\psi 40$, lane 10; $\psi 52$, lane 11.

Fig. 4 Comparison of transcription from promoters in intact spacers and from gene promoters after injection into oocytes.

Transcription from both types of promoter was detected by primer extension analysis of aliquots of RNA from injected oocytes using two different primers. Lanes 1 through 4 show the signal from spacer promoters on pXlr409, 264, 164 and 101A respectively, after injection of equimolar amounts of plasmid into *X. borealis* oocyte nuclei (*X. borealis* oocytes were used to avoid cross-hybridisation of probes to endogenous transcripts). Lanes 5 through 8 show the transcription signal from the gene promoters on pXlr409, 264, 164 and 101A respectively. Note that pXlr264 has no gene promoter and thus gives no signal band in lane 6, and that the weaker gene signal from pXlr 409 in lane 5 is a feature of mini-gene constructs in comparisons with intact genes by primer extension analysis. The spacer signal is complex because the probe hybridises to the spacer transcript at several positions.

Fig. 5 Measurements of endogenous spacer and gene promoter activity in oocytes, embryos and cultured cells.

Transcription from both types of promoter was measured using the same primer extension probes used in Fig. 4.

a. lane 1 gene promoter signal from five *X. laevis* oocytes; lane 2 gene promoter signal from 25µg of cultured kidney cell RNA; lane 3 spacer promoter signal from five *X. laevis* oocytes; lane 4 spacer promoter signal from 25µg of cultured kidney cell RNA.

b. lane 1 gene promoter signal from five *X. laevis* embryos, early neurula stage; lane 2 gene promoter signal from 25µg of cultured kidney cell RNA; lane 3 gene promoter signal from five *X. laevis* oocytes; lane 4 spacer promoter signal from five *X. laevis* embryos, early neurula stage; lane 5 spacer promoter signal from 25µg of cultured kidney cell RNA; lane 6 spacer promoter signal from five *X. laevis* oocytes; lane 7 pBR322/HpaII molecular weight markers.

our kidney-derived tissue culture cell line showed a high level of spacer promoter activity that was about equal to the activity of the gene promoter (Fig. 5a, lanes 2 and 4; Fig. 5b, lanes 2 and 5). Moss has also detected some spacer transcription by S_1 assays, in his tissue culture cell line (9). It seems then, that, using a biochemical assay for promoter activity, plasmid-borne spacers injected into oocytes behave like the endogenous spacers of tissue culture cells rather than the endogenous spacers of oocytes.

DISCUSSION

In this study we have shown that the variable levels of rDNA spacer transcription observed among oocytes in vivo are unlikely to be explained by DNA sequence differences between spacer promoters. Moreover, it seems that the usual inactivity of spacer promoters relative to gene promoters that is observed in oocytes in vivo can not be explained simply by the 10% sequence difference between the two types of promoter. This conclusion stems from our primer extension assays of plasmid-borne spacer and gene promoters competing in the same oocytes, which show that the activities of the various promoters are equivalent. A parallel situation may exist in *Drosophila*, where rDNA repeats that contain an insert in the 28S rRNA gene are not expressed in vivo despite their promoters' being identical in sequence to those of expressed intron-less genes (18).

Our results have raised two further puzzles:

- 1) The fact that endogenous spacer promoters in oocytes are usually only slightly active or silent has been demonstrated both by biochemical and electron microscope procedures (4-6). However, our biochemical measurements of abundant spacer transcription on injected plasmids are at variance with a number of electron microscope observations of these very same plasmids that we and others have reported previously (8,10,17). For example, pXlr164 and pXlr264 regularly show some spacer transcription in electron microscope assays, whereas pXlr101A does not. However, primer extension (Fig. 4) and S_1 nuclease protection assays (data not shown) have revealed abundant and equivalent amounts of spacer transcription on all three plasmids. While we are confident that both types of assay are a measure of initiation frequency, we think that the different results obtained indicate that transcription initiation on plasmids injected into oocytes may proceed via two different pathways. Electron microscope assays may detect only a particular sub-population of transcribing plasmids, namely those that reflect the endogenous situation of genes densely packed with polymerases and transcripts. Conversely, in terms of mass, most of the spacer and gene transcripts in

injected oocytes may be derived from a much larger population of plasmids on which all promoters initiate transcription infrequently. These plasmids might be extremely under-represented in electron microscope assays because they bear only a few polymerase molecules per plasmid.

2) A second puzzle concerns the finding that spacer promoters on plasmids injected into oocytes are at least as active as injected gene promoters when assayed by primer extension, whereas the endogenous spacers of oocytes show almost no promoter activity by the same assay. Recently we have found that the spacer promoters on injected plasmids or in tissue culture cells can be selectively inactivated by treatment with intercalating agents such as ethidium bromide or chloroquine (S.C. Pruitt and R.H. Reeder, manuscript in preparation). This finding raises the possibility that differential super-coiling may be the cause of the differences in spacer transcriptional activity that are observed in different situations.

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