Upstream sequences required for transcription of the TFIIIA gene in *Xenopus* oocytes

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Received January 14, 1988; Revised and Accepted April 7, 1988

ABSTRACT

The DNA sequences required for efficient initiation of transcription of the *Xenopus* transcription factor IIIA (TFIIIA) gene were determined by microinjecting a series of deletion and linker substitution mutants into *Xenopus* oocyte nuclei. An upstream activating sequence, which resides between residues -283 and -238, and perhaps a second sequence between -167 and -122 preceding the transcription start site, together stimulate transcription about 30-fold. The distance between the two sequences and the distance from them to the initiation site can vary by at least 13 base pairs without loss of activity. The TFIIIA upstream sequences can stimulate transcription of other genes, for example, they stimulate transcription from the herpes thymidine kinase promoter about 30-fold.

INTRODUCTION

The *Xenopus* 5S RNA genes require the transcription factors TFIIIA, B and C in addition to RNA polymerase III for transcription (1,2). TFIIIA, a 40,000 dalton protein, binds to the internal control region of 5S RNA genes (3,4,5) and independently associates with the two other transcription factors to form a stable transcription complex (6).

Xenopus has two classes of 5S RNA genes, oocyte type and somatic type, which are differentially expressed throughout development (7,8). The oocyte type 5S RNA genes are expressed only in oocytes, while the somatic type genes are transcribed both in oocytes and in somatic cells (9,10). The cellular content of TFIIIA protein is also developmentally regulated (11). In immature oocytes it is present at about 2×10^7 molecules per 5S RNA gene, but falls to less than one molecule per gene in somatic cells. This correlation between the level of TFIIIA and the expression of oocyte type 5S RNA genes leads to the hypothesis that the developmentally regulated expression of TFIIIA may control the differential expression of 5S RNA genes (12,13). The precise mechanism remains to be determined by which the concentrations of TFIIIA and of other transcription factors, the

differential affinity of TFIIIA for the two types of 5S genes (14,15) and the temporally ordered replication of the two types of 5S RNA genes (16,17,18) contribute to the more than 1000-fold preferential transcription of somatic type over oocyte type 5S genes in somatic cells. Nevertheless, the observation that the addition of purified TFIIIA protein increases the expression of the 5S RNA gene in an in vitro transcription system with somatic cell extracts (19) suggests that the level of TFIIIA is a factor in regulating 5S RNA expression through development.

Recently cDNA and genomic DNA clones of TFIIIA have been isolated (20,21,22). Analysis of the TFIIIA mRNA showed that its level correlates well with the level of TFIIIA protein throughout development (20), suggesting that the expression of TFIIIA is regulated at the transcriptional level. Analysis of the TFIIIA genomic clone revealed a CCAAT box at -94 and a TATA box at -32 nucleotides from the transcription initiation site (21). It is possible that these sequences are involved in regulating transcription of the TFIIIA gene.

In this study, we determined the sequences required for efficient initiation of transcription of the TFIIIA gene in oocytes. We found that two sequences upstream of the CCAAT box are required for efficient transcription. The distance between the two sequences as well as the distance from them to the start site can be varied somewhat without loss of function. Furthermore, the upstream activating sequences can act to stimulate transcription of other genes.

MATERIALS AND METHODS

DNA Constructions

pTF-G1 was constructed by ligation of two EcoRI fragments (6.5 kb and 8.6 kb) derived from the Xenopus genomic TFIIIA clone λ XLGTF1 (21). pTF-G1 Δ was obtained by deletion of a HaeII fragment (+65 to +114) from the first exon of pTF-G1. In pTF-CA, a 1.1 kb PstI fragment derived from a Xenopus TFIIIA cDNA clone was substituted for the DNA from the PstI site in the first exon to the PstI site in the last exon of pTF-G1 Δ . pTF-CA-424 was constructed by deletion of a 1.4 kb EcoRI-BglII fragment from pTF-CA. These structures are shown schematically in Fig. 1.

A series of deletions were generated by the method of Guo and Wu (23). For 5' deletions, pTF-CA-424 was digested with exonuclease III and S1 nuclease from the BglII site at -424 bp toward the coding region, treated with the Klenow fragment of DNA polymerase I and ligated to a 10 bp HindIII

linker (GCAAGCTTGC). After digestion with HindIII and XhoI (at +722 of the coding region), the resultant deletion DNA fragments were inserted into M13mp10 for sequencing. The fragments on M13 were then cut with HindIII and BglII (at +353) and subcloned into pTF-CA. For 3' deletions pTF-CA was digested from the PstI site at +139 bp toward the upstream region and treated in the same way as for 5' deletions. The deletion fragments were cut with HindIII and DraI (at -0.6 kb) and inserted into M13mp10. After sequencing, these cloned deleted fragments were then recombined at the HindIII site with some of the 5' deletion mutants to generate linker substitution mutants (24). The mutants are named by the position of the last remaining residue of TFI_{IIA} DNA; residue +1 is the putative transcription initiation site (21). For example, LS-118/-122 has a HindIII linker joining TFI_{IIA} upstream residues -118 of a 3' deletion mutant and -122 of a 5' deletion mutant, consequently it contains a 5 bp overlapping sequence (-122 to -118) separated by a HindIII linker. Diagrams of these mutants are shown in Figs. 3A and 4A.

To construct hybrid genes of TFI_{IIA} and the herpes simplex virus thymidine kinase gene (tk), a TFI_{IIA} 3' deletion mutant, Δ -75, was ligated to a 5' deletion -37 of the tk gene (24), using the HindIII-BamHI fragment derived from the polylinker of pUC8 as a conversion adaptor. The detailed structures are shown in Fig. 5A.

Microinjection and S1 Nuclease Mapping

Microinjection into X. laevis oocytes was performed as described previously (25). Approximately 20 oocytes per DNA sample were injected with 0.15 fmol each of the indicated deletion plasmid and the control plasmid pTF-G1. After incubation at 19°C for about 20 hours, total RNA was extracted from the injected oocytes (26) and four oocyte equivalents of RNA were analyzed by S1 nuclease mapping.

S1 nuclease mapping was performed essentially according to Tso et al. (27). The three single-stranded DNA probes used in this work were labeled uniformly with [α -³²P]CTP. The probe used for analysis of TFI_{IIA} mutants (probe 1, see Fig. 1) was prepared with a synthetic oligonucleotide primer, TATAAGCAGCGCCGC (+124 to +110 of the TFI_{IIA} coding strand; prepared using the Beckman DNA synthesizer), and the M13mp10 single-stranded DNA containing the BglII-PstI fragment from -424 to +144 of the TFI_{IIA} gene. This probe gives a signal of 124 nucleotides for the transcript from the control plasmid and a signal of 70 nucleotides (from the transcription start point up to the intragenic deletion) from each deletion mutant in S1 nuclease

mapping. To detect tk transcripts a second probe was used. It was prepared with the sequencing primer -40 (New England Biolab cat #1212) and the M13mp10 single-stranded DNA carrying the 0.2 kb EcoRI-RsaI fragment of the tk gene. This probe covers the region from -80 to +119, giving a signal of 119 nucleotides for the tk transcript. In these experiments another probe was used to detect the control TFIIIA transcript. It was prepared with the same sequencing primer and the TFIIIA-cloned single-stranded DNA used above. This probe gives a signal of 144 nucleotides, which is readily distinguishable from that for the tk transcript in S1 nuclease mapping. The intensity of the autoradiographic bands in S1 nuclease mapping was determined by densitometry within the linear range and divided by the number of labeled C residues in a protected fragment (28, 39, 33, and 49 labeled C residues in the 70, 124, 119, and 144 nucleotide bands, respectively). The relative activities of mutants to the control are shown in a histogram.

RESULTS

5' Flanking Deletion Mutants

The starting material for the 5' flanking deletion mutants used in this study, pTF-G1 (Fig. 1) is the ligated DNA from the two EcoRI fragments (6.5 kb and 8.6 kb) derived from the Xenopus laevis genomic TFIIIA clone λ XLGTF1 inserted into pEMBL8+ at the unique EcoRI site (21). This DNA contains 5'- and 3'- flanking sequences of about 2 kb each in addition to the intact exons and introns of the TFIIIA gene (except the fifth intron, from which a 46 bp EcoRI fragment was deleted). We injected this DNA into the nuclei of Xenopus oocytes and assayed its transcriptional activity by S1 nuclease mapping analysis (Fig. 2, see upper portions of lanes U and 1). pTF-G1 directed transcription in oocytes (shown by a 124 nucleotide long protected band) and its initiation site is consistent with that of TFIIIA mRNA reported by Ginsberg et al. (20). The transcription from the injected DNA gave a much stronger signal than the endogenous oocyte TFIIIA transcription, which can be seen only in longer exposures. The strong transcription is probably due to the high number of injected plasmid DNA molecules compared with the endogenous TFIIIA gene.

To localize the sequences essential for efficient initiation of transcription, we generated deletions in TFIIIA DNA and analyzed their transcriptional activities by injection into oocytes followed by S1 nuclease mapping of the RNA. We distinguished the transcript of an injected DNA from the endogenous transcript by introducing a second, small deletion into the

Fig. 1. Diagram of TFIIIA DNAs. The boxed regions denote exons or coding regions. +1 and (A)_n indicate the transcription initiation site and the polyadenylation site, respectively. Δ, a 49 bp *Hae*III deletion in the first exon of the TFIIIA gene. Probe 1 used for S1 nuclease mapping is also shown at the top.

first exon of all the TFIIIA deletion mutants (see Fig. 1, and Materials and Methods). As a result of this small deletion, transcripts derived from these mutant plasmids gave a protected band of 70 nucleotides in length. While transcripts from the endogenous TFIIIA gene as well as pTF-G1 gave a signal of 124 nucleotides, the intragenic deletion (pTF-G1Δ) shortened the signal but did not affect transcriptional activity (Fig. 2, lane 1 - taking into account the relative lengths of the uniformly labeled, protected probe). In all the injection experiments described below we coinjected pTF-G1 with the deletion mutants and used pTF-G1's activity as a standard to normalize transcriptional activities of the deletion mutants.

First, we deleted all the introns in the TFIIIA gene by substitution of TFIIIA cDNA for the region from the first to the last exon (pTF-CΔ). In addition, we also shortened the 5' flanking region to 424 bp (pTF-CΔ-424). These two constructs were transcribed at similar levels to the original one (Fig. 2, lanes 2 and 3). These results suggest that none of the introns nor the upstream sequence beyond -424 are necessary for transcription of TFIIIA in oocytes.

The 5' flanking region of many eukaryotic genes contains sequences required for efficient transcription; e.g. UAS of yeast genes (28,29,30), and the herpes simplex virus tk gene (24). To determine if this is also the case for the TFIIIA gene, we introduced a series of deletions into the 424 bp 5' flanking sequence. In one set of deletion mutants, TFIIIA DNA was progressively shortened from the 5' terminus of the flanking sequence toward

FIG. 2. Transcription of TFIIIA DNA in *Xenopus* oocytes. lane U, uninjected; lane 1, pTF-G1 Δ ; lane 2, pTF-CA; lane 3, pTF-CA-424, lanes 1-3, were coinjected with a control DNA, pTF-G1. The transcripts were analyzed by S1 nuclease mapping and electrophoresed on a denatured 8% polyacrylamide gel. 124 and 70 indicate the position (in nucleotides) of transcripts from the control plasmid, and from the DNAs containing the 49 bp *Hae*II deletion, respectively. The relative transcriptional activities of the mutants are shown in the histogram below each lane (see Materials and Methods).

the initiation site of transcription (5' deletion mutants: Fig. 3A). As shown in Fig. 3B, deletion mutants Δ -424 to Δ -283 (lanes 1 to 6) showed comparable transcriptional activity (see histogram at the bottom of figure). In Δ -254, however, the activity was significantly reduced. Further

FIG. 3 Transcription of 5' deletion mutants. (A) Diagram of mutants. Solid lines indicate TFIIIA DNA remaining after deletion. Δ -, position of 5' most base pair of TFIIIA DNA. (B) S1 nuclease mapping with these mutants (see legend to Fig. 2). The lane numbers correspond to the 5' deletion plasmids in (A). The band seen at the top of lane 13 resulted from non-specific transcription initiated in the plasmid vector DNA.

FIG. 4. Transcription of linker substitution mutants. (A) Diagram of mutants. The system for naming mutants is described in Materials and Methods. Solid lines indicate TFIIIA DNA remaining in mutants. In the mutant R-118/-122, the orientation of the DNA between -424 and -118 has been reversed. The change in distance caused by the mutation is shown in the right column. I and II indicate the first and second upstream activating sequences (see text). (B) S1 nuclease mapping with these mutants. The numbers above the lanes correspond to the numbers in (A) (see legend to Figs. 2 and 3).

deletions, Δ -232 to Δ -75, lead to very low transcription activities. Similar result was obtained from a second experiment with these mutants. Although the transcriptional activities are extremely low in these mutants, the start site of transcription remains the same, as seen in longer exposures. The results shown here suggest that the TFIIIA gene has at least one upstream activating sequence in addition to the TATA and CCAAT boxes and that its 5' border is located between -283 and -254.

We see minor variations in activity from similar deletions. Since these minor variations are within experimental error, we chose to focus on the mutants that caused major differences in activity, the effect of which was approximately the same in different experiments.

Linker Substitution Mutants

To define more precisely the boundaries of the major upstream activating sequence, we used a set of linker substitution mutants. In these constructs the 5' flanking sequence was deleted in a 3'-5' direction from the PstI site in the first exon, and the resultant deletion mutants were ligated to some of the 5' deletion mutants using the HindIII site introduced at the end point of each deletion.

As shown in Fig. 4B, LS-271/-254 (lane 2) had minimal activity and LS-258/-232 (lane 3) had no activity while LS-238/-232, -229/-218, -206/-208, -196/-167, -167/-167 (lanes 4 to 8) and the positive control, Δ -424 (lane 1), all showed substantial activity (ratio of at least 0.4). The regions deleted in LS-271/-254 and -258/-232 are close to the upstream activating sequence determined in the 5' deletion experiments. The results also suggest that the 3' border of the activating sequence is between -258 and -238 (compare lanes 3 and 4).

Another mutant, LS-167/-122 (lane 9) was at least 10-fold less active than its neighboring mutants. The decrease in activity could have been caused by deletion of a second critical sequence or a change in the spatial relationship of the first activating sequence with the initiation site. In this mutant, the distance between the upstream activating sequence described above and the transcription initiation site was shortened by 34 nucleotides. However, we observed that the construction in which the region from -238 to -122 was replaced by a M13 DNA fragment to maintain the same spatial length resulted in a loss of transcriptional activity. In a second experiment with these mutants similar results were obtained. This observation supports a second transcription activating sequence between -167 and -122.

In other experiments, we used a mutant in which the upstream region

spanning -424 to -118 was reversed in orientation and ligated to the 5' deletion Δ -122 to determine whether the upstream region of TFIIIA has orientation independent "enhancer" activity. An enhancer is generally defined as a sequence which stimulates transcription independently of its

FIG. 5 Transcription of hybrid TFIIIA and herpes simplex thymidine kinase genes. (A) Diagram of hybrid genes. ds-1 and ds-2 indicate the distal signals required for transcription of the tk gene (24). -37 refers to the position of tk DNA, whereas -75, -181 and -0.6 kb refer to the positions of TFIIIA DNA. (B) S1 nuclease mapping with the indicated DNAs was performed as described in the legend to Fig. 2. 144 indicates the signal of the transcript from the control plasmid, pTF-G1 (the probe used in this experiment is different from the previous one. see Materials and Methods.), and 119 indicates the signal from the tk coding region. The band near the top of lane 3 comes from non-specific transcription initiated in the plasmid vector DNA.

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-350      GCATCCATCACATTCAACAGTTACAGTTTCTCCAACACCAGCAGCTGC
-300      TGCACACCGTTTCCTCGGCTTCATGTATTATCACGTGCTCCACTAGGACT
-250      CAACCACTAAGAACGAGGGGAGTGTCAGAAACACCCAACTTGTGAAATA
-200      ACACATCGCTGACATAAAACACAGGAATTTAACATCCTTTTTTTTAAAGTT
-150      TGCGAGCGCAATTACTGAAGGCTAAAGCTGCAAGGGACACAGGAAAGGAC
-100      TGATTGCCAATCCTTTCAGACATCGCAAACTTCCCGATGCATGTGCGAT
-50       AATGGTTTGTCTAGAGCTATATAAACAGGCACACATGGCGGCTACAGTG      -1

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FIG. 6 The sequences required for efficient initiation of transcription of the TFIIIA gene. Two upstream activating sequences as well as the CCAAT and TATA boxes are underlined.

orientation and location (31). As shown in lane 11 of Fig. 4B, the TFIIIA upstream sequence in the reverse orientation did not stimulate transcription at all, though the same sequence functions to activate transcription when placed in the original orientation (LS-118/-122, lane 10 in Fig. 4B). When the upstream region from -424 to -238 was reversed in orientation and ligated to Δ -232, it was also transcriptionally inactive (date not shown). These results suggest that at least one of the TFIIIA upstream sequences discussed above does not function as an enhancer.

TFIIIA/tk Hybrid Genes

Taken together, the results presented above demonstrate that the upstream region contains at least one and possibly two sequences in addition to the CCAAT and TATA boxes required for transcription of the TFIIIA gene. We have also investigated 1) whether the coding region is required for transcription initiation and 2) whether the upstream activating sequences for the TFIIIA gene can function to stimulate transcription of other genes. To determine whether the TFIIIA coding region is necessary for the stimulation of transcription initiation by the upstream sequence, we constructed hybrid genes of the TFIIIA upstream sequence, and the TATA box and coding region from the herpes simplex virus tk gene, and examined their transcriptional activities. The tk gene has two upstream distal signals in addition to the TATA box, all of which are necessary for efficient

transcription in *Xenopus* oocytes (24). As shown in Fig. 5, one of the hybrid genes, in which the essential TFIIIA upstream sequences and the CCAAT box were ligated to the TATA box and coding region of the tk gene, showed transcriptional activity (lane 3), while the tk gene was not transcribed when only the second upstream sequence and CCAAT box of TFIIIA were present (lane 2). This result suggests that the TFIIIA upstream sequence can stimulate transcription independently of the coding region. Furthermore, it shows the ability of these sequences to stimulate transcription of other genes.

DISCUSSION

We used a series of deletion and linker substitution mutants to analyze sequences required for efficient initiation of transcription of the TFIIIA gene in *Xenopus* oocytes. The analysis revealed that the upstream flanking region is necessary and sufficient, and that the coding region, introns, and 3' flanking region are dispensable for transcription initiation. We also demonstrated that the upstream flanking region contains one or possibly two transcription activating sequences in addition to the CCAAT and TATA boxes; the first sequence lies between -283 and -238, and a possible second sequence lies between -167 and -122. These sequences are shown in Fig. 6. The distance between them can be varied, at least moderately, without loss of function. LS-206/-208 and -196/-167, in which the distance between the two upstream activating sequences is altered by +13 bp and -18 bp, respectively, and LS-118/-122, in which the distance between the second sequence and the CCAAT box is altered by +15 bp, still maintain transcriptional activity. The transcription initiation site of the TFIIIA gene is unaffected even when the distance between the upstream activating sequences and the coding region is changed. This is probably due to the TATA box at -32 (32). In a hybrid gene pTF-tk2 (Fig. 5B lane 3) the transcription initiation site also appeared to be directed not by the upstream activating sequences and CCAAT box, but by the TATA box of the tk gene. These observations suggest that the TATA box also plays a role in transcription initiation of the TFIIIA gene.

Deletion into the two activating sequences led to almost complete loss of transcriptional activity (Fig. 4B lanes 2,3 and 9). This suggests that both upstream activating sequences are required for stimulation of transcription. Our observation that the TFIIIA upstream activating sequences can stimulate transcription of the herpes tk gene suggests that

the sequences are not specific for the TFIIIA gene but can function with heterologous promoters.

The two upstream activating sequences do not contain repeated sequences or dyad symmetries. We have not detected any sequence similarities with a number of other transcription activating sequences reported previously. Ginsberg et al. (20) discovered sequence similarities between the TFIIIA coding region and the 5S RNA and tRNA^{met} genes, suggesting the possibility that these sequences may be required to regulate TFIIIA expression. We demonstrated here that the upstream flanking sequence of TFIIIA stimulated transcription even when its coding region was replaced with the tk coding region, and its activity was comparable with the activity in the original TFIIIA gene. Thus, the sequence similarities observed by Ginsberg et al. (20) are probably not necessary for efficient transcription of the TFIIIA gene in oocytes.

Possible mechanisms for developmental regulation of TFIIIA expression include 1) a positive factor for transcription that is abundant in immature oocytes but decreases through development or 2) a negative factor which increases through development. The transcriptional activating sequences in the TFIIIA upstream flanking region identified here are good candidates for binding sites of some trans-acting positive factor(s). Conversely we did not find any obvious evidence for negative control by our analysis. Nevertheless, the possibility of negative control of TFIIIA cannot be excluded because hypothetical negative factor(s) may be titrated out by higher copies of injected TFIIIA DNA or be absent in mature oocytes. For elucidation of the precise mechanism of TFIIIA regulation, similar TFIIIA gene mutant analysis in somatic cells will be helpful.

ACKNOWLEDGMENTS

We thank L. Ho and C. Siebel for microinjection of *Xenopus* oocytes; D. Guinta, J. Tso and N. Tsurushita for many helpful discussions; D. Bogenhagen, C. Queen, C. Siebel, J. Tso and N. Tsurushita for comments on the manuscript; J. Ohms and J. Burns at Beckman Instruments for synthetic oligonucleotides; S. McKnight for the tk gene plasmid; and J. Ward for excellent secretarial assistance.

This work was supported by grants from the National Science Foundation (PCM-8402553) and the March of Dimes Birth Defect Foundation (1-915) to L. J. K. L. J. K. was an American Cancer Society Junior Faculty Research Awardee during part of this work.

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