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An enhancer responsible for activating transcription at the midblastula transition in *Xenopus* development

(egg/microinjection)

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ABSTRACT **Transcripts from the *Xenopus* gene GS17 are absent from oocytes and first appear at the midblastula transition (MBT), about 8 hr after fertilization. Injection experiments with deletions of the GS17 gene and GS17-β-globin hybrid genes show that a relatively short sequence of 74 base pairs is sufficient to activate, in cis, transcription at the MBT. This 74-base sequence is located about 700 bases upstream of the GS17 promoter and has the properties of an enhancer element.**

The events of early development are regulated by gene products encoded by the maternal and embryonic genomes. In *Xenopus*, maternal components are responsible for development throughout cleavage and up to the 4000-cell stage, when transcription of the embryonic genome suddenly commences (1, 2). This transcriptional activation is one of the events that characterizes the midblastula transition (MBT) (3–5). Most of the RNA synthesized at the MBT is 5S RNA, tRNA, and small nuclear RNA, which are already present in the egg, but some embryo-specific RNAs are also transcribed at this time (6, 7). Inhibition of transcription at the MBT prevents gastrulation (8, 9), and so presumably some of the new embryonic transcripts play important roles in early development. The aim of our current research is to determine the mechanism by which some, but not all, embryonic genes are selected for transcription at the MBT.

In several organisms the mechanism of specific gene activation during early development has been investigated by injecting altered genes into developing embryos. For example, developmentally regulated genes have been studied in *Drosophila* (10, 11), mice (12, 13), and sea urchins (14). Similarly, we have studied transcriptional regulation in *Xenopus* development by microinjecting an embryonic gene into fertilized eggs.

The gene that we use in these expression experiments, GS17, is among the very first to be expressed by the embryonic genome. GS17 mRNA first appears at the MBT, reaches maximum levels at gastrulation, and then rapidly disappears during neurulation (7). We infer from the fact that GS17 comes on precisely at the MBT that its selection for transcriptional activation is controlled by maternal factors. The function of the 17,000-dalton protein encoded by the GS17 mRNA has not been determined. When cloned copies of the GS17 gene are injected into fertilized *Xenopus* eggs, transcription of the injected gene is correctly regulated during subsequent development—i.e., the switch-on and switch-off of transcription occurs at the same time for the injected and the endogenous genes (7). The GS17 plasmid used in those studies contained 2000 bases of the 5′ flanking region of GS17 and we therefore concluded that the signals required for correct developmental expression must be located within this region. By extending these simple expression experiments we have now analyzed the 5′ flanking region of GS17 in more detail and identified a transcription enhancer sequence that is located ≈700 bases upstream from the GS17 promoter. The experiments reported here suggest that this enhancer sequence is necessary for the transcriptional activation of GS17 at the MBT.

MATERIALS AND METHODS

Plasmids and Production of Deletion Constructions. The plasmid p2.0B is derived from pGS17 5′ (7). It contains the same fragment of the GS17 gene (about 2000 bases of the 5′ flanking sequence and 120 bases of the first exon), cloned into pSP64 (15), but is marked at the 3′ end by a 29-base *Eco*RI–*Hin*dIII fragment of pBR322. Plasmid p2.0B was the starting material for the construction of the 5′ deletion series. Deletions that removed increasingly longer pieces of the 5′ flanking region of the GS17 gene were generated by using a DNase I/$MnCl_2$ procedure (16), and the resulting plasmids were sized on 1% agarose gels. Plasmids containing end points spaced at about 200-base intervals along the GS17 sequence were selected for expression analysis. The names of the deletion plasmids contain the length [in kilobases (kb)] of the 5′ flanking region retained in each construction (see Fig. 1).

For analysis of enhancer properties, fragments of GS17 DNA were isolated from the deletion plasmids. The *Eco*RV site at −605 or the *Dra* I site at −681 marked the 3′ ends of the excised fragments (see Fig. 2). *Eco*RI linkers were added to the *Eco*RV or *Dra* I ends of the GS17 DNA fragments and following *Eco*RI cleavage the enhancer fragment was inserted into the *Eco*RI site of pXβ (7). pXβ contains the entire *Xenopus* adult β-globin gene (17), including 471 bases of 5′ flanking sequence containing the β-globin promoter.

DNA Sequence Analysis. The DNA sequence of the 5′ flanking region of the GS17 gene was determined by using the chain-termination procedure of Sanger *et al.* (18). Overlapping sequences were obtained by cloning inserts from the deletion constructions into M13 sequencing vector mp10 or mp11 (19).

Injection of Fertilized Eggs. *Xenopus laevis* females were obtained from Xenopus I (Ann Arbor, MI). Injection and incubation of the fertilized eggs were carried out as described (7). All embryonic stages refer to the timetable of Nieuwkoop and Faber (20).

RNA Preparation and Analysis of Transcripts. Total RNA was isolated from embryos by proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation as described (21). Prior to use in protection experiments, RNA was separated from other ethanol-precipitable material by precipitation with 4 M LiCl. The 5′ ends of gene transcripts were mapped using an RNase protection assay as described

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Abbreviations: MBT, midblastula transition; kb, kilobases; bp, base pairs.

(15). In each case appropriate restriction fragments containing the 5′ end of the gene were cloned into the polylinkers of pSP64 or pSP65 (15). Uniformly labeled RNA probes (6×10^8 dpm/μg) were synthesized from linear templates using SP6 polymerase as described (15), except that the reactions were performed at 30°C. Under limiting nucleotide concentrations, incubation at 30°C can result in a greater yield of full-length product (22). The sizes of full-length probe and of the expected RNase-protected fragments are shown in each figure.

RESULTS

Deletion Analysis of the GS17 Promoter. Previous experiments have shown that a 2-kb region of DNA at the 5′ end of the GS17 gene is sufficient to provide correct regulation of transcription during early development, from the blastula to neurula stage (7). To identify the DNA sequences that are required for the transcriptional regulation of GS17, we began by examining the expression of a series of deletion constructions (see *Materials and Methods*). The deletion constructions used in these studies contain 5′ flanking regions ranging from about 1660 bases to 240 bases. These constructions are illustrated in Fig. 1*A*. All constructions contain a short marker sequence so that transcripts from the injected and endogenous GS17 promoters may be distinguished.

Fifty picograms of each DNA to be tested was injected into fertilized *Xenopus* eggs as supercoiled plasmid DNA and transcription from the GS17 promoter was assayed by RNase protection analysis. Total RNA from injected embryos was assayed at early gastrula (stage 10) when endogenous levels of GS17 mRNA are near maximum. The results of the RNase protection experiment are shown in Fig. 1*B*. The amount of endogenous GS17 RNA (protected fragment, 116 bases in length) is approximately equal in all tracks, indicating a reproducible recovery of RNA from the injected embryos. The amount of marked transcript derived from the different deletion constructions, however, shows dramatic variation. Though the amounts of transcripts produced by the first six constructions (p2.0B to p0.93D) are approximately equal to each other and similar to the amounts synthesized by the endogenous gene, no marked transcripts are detected in RNA isolated from embryos injected with deletion construction p0.70D, p0.60RV, or p0.24Sc. Southern blot analysis was carried out to determine the amount of plasmid template DNA contained in the injected embryos. These data (not shown) reveal that the amount of template is about equal in each sample, although variations in template concentration do account for the slight variation in amount of marked transcript observed in Fig. 1*B*. For example, about a 2-fold amplification of the amount of p1.66D template DNA is responsible for the increase in marked transcript in track 2. Despite these small variations, the amount of DNA present for p0.70D, p0.60RV, and p0.24Sc (tracks 7–9) does not differ significantly from that of the longer constructions (tracks 1–6). Therefore, the major decrease in the amount of transcription from the three shortest deletion constructions does not correlate with a significant decrease in the amount of template DNA. We conclude that a sequence required for efficient expression of the GS17 gene has been removed in p0.70D. This places a critical sequence at least 700 bases upstream of the cap site of the GS17 gene.

DNA Sequence of the 5′ Flanking Regions of the GS17 Gene. To help identify and characterize the transcriptional control elements, the DNA sequence of 1000 bases of the flanking sequence of the GS17 gene was determined (see Fig. 2). RNase protection experiments have suggested that GS17 mRNA consists of several species that differ slightly in the length of their 5′ untranslated regions (7). The residue marked as +1 in Fig. 2 corresponds to the cap site of the longest but least abundant of these mRNA species. Inspection of the DNA sequences shows that "TATA" and "CATT" boxes are located at the expected positions relative to the cap site, at approximately −30 and −80, respectively. The region of DNA determined to be essential for efficient transcription in the deletion analysis experiments is at least 700 bases upstream of these common promoter elements. This suggested that the upstream region might contain a transcriptional enhancer sequence.

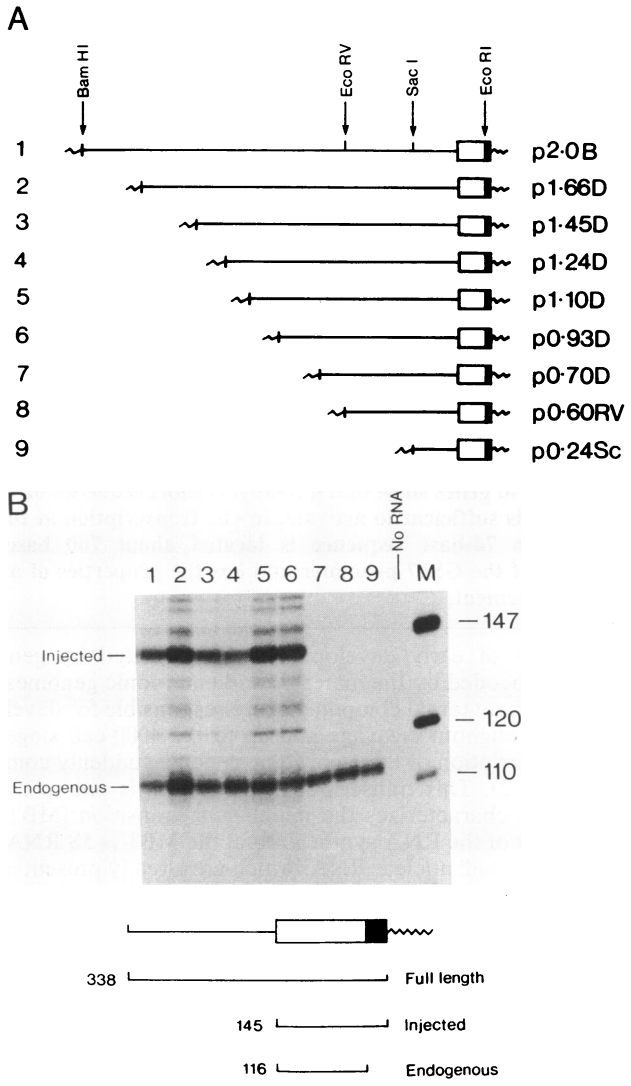

FIG. 1. Expression of GS17 deletion constructions in injected embryos. (*A*) Structure of the deletion plasmids. Solid black line represents the 5′ flanking region of the GS17 gene. The open box represents a portion of the first exon of the GS17 gene and the black box indicates a marker sequence used to distinguish transcripts from the injected and endogenous genes. pSP64 sequences are shown as ∿. The names of the plasmids indicate the length of GS17 5′ flanking sequence contained in each construction. (*B*) Fifty picograms of supercoiled DNA was injected into fertilized eggs as the first cleavage began. Injected eggs were allowed to develop to early gastrula, stage 10, and total RNA was extracted for transcript analysis. Transcripts from the endogenous and injected GS17 genes were detected by RNase mapping using single-stranded SP6 RNA probes. RNA from one embryo equivalent was assayed and the protected fragments were fractionated on a 6% acrylamide/8.3 M urea gel. The full-length probe and the fragments protected from RNase digestion are shown below the autoradiogram. Numbers above each track correspond to the plasmid DNA injected. Size markers (M) are *Hpa* II fragments of pBR322.

Enhancing Activity of GS17 Sequences on a Heterologous

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-960
   TGATCAAAACAATACACAAGCCCTATTTATTGCACGGAGATTGAAAGGGGTGGGGTGCAC
-900
   CGTGCTCCCAGTTTAATTAGCATGACTGGGAAAGTGTTTGTGACCCCCTTTAATTGCTAA
-840
   TGAAGCCCATGAGGGCTCAATTGTTGTTGCAGGAGACGCAAGAGACACACCCAACAAGGA
-780
   AAACAAAAAGTCCTGCTAGTCTATAGGGGGTCCTTCCTCAGTTTTGTGTCCCCCTCTCAG
-720                    Dra I
   GTGTCACAGGCCCCCTGGCCCCTCCCCTTTGTTCCATTTAAATACCTGGTTAAATAACCC
-660                                                  Eco RV
   ATCTGGATCAGAACACAGAGCAGAAGCTGAGAAGCAGCAGAAATCGTGGTATGTATACTT
-600
   TACATTTATCTTTGCTTATGGAAATATGAGAATGATAGGATATCCTGAGCTTGCATTTGA
-540
   AATGTGATTTTGCCTGTTTGGTGGGGGTCACTGACCAGCTGTCGGTACAAATGTAGTTAT
-480
   TTTTTTTACTACTTATTTTTATTCCAGTCTGTCATTAAAAACAATGCCTTTGCTAGGGTA
-420
   AACGGGACCCAATCAACCACCCACTAGAAATACTAACACATTACATCTTACTTTATAAGG
-360
   CATTAGGGGCAGTTAGATGAAGTATTAGTTGGCGGATATATTGGGTTATATATTCTGTTT
-300
   AGATAAAGACACATTTTAAGGGGAAGTTATTTATGGGGTAAGTTTTAACAGACTCACAGA
-240 Sac I
   GAGCTCCTTCCATGTCCCCCAAGCTCTAAACAATGGCTCACGCAGTAGTTTGAGTGGCAG
-180
   CTGGTGAGCAAGTGCCCCAACAGGCACAAAACGTGCCAGGCCTTCACCTTTATGTCCTAA
-120      Dra I
   TAAATGGTTTAAACTTTTAATTATATTTTTGGTCATTTCTCTTAACTGCTGGTGGGTACA
 -60                                                        -1
   GACTGGTTATACCCATACTGAACACATATTAATACACACATTTCAATTATTGGTTTTGTA
 +1                                                         60
   GATAAAAGAAACAGACTCCAGTTATGTCCCAGAATTTGGATTTCTTGGCCTTGGCTGGCC
                                                             120
   GTGGAGGTCCTCATTATGCCAGTCCAACTTCAAGGCATCCCTCACCAAAGGTTTGGAATT
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FIG. 2. DNA sequence of the 5′ flanking region of the GS17 gene. The sequence is numbered from the cap site of the gene, and the TATA box, CAT box, and initiation codon are underlined. A vertical arrow marks the end point of deletion p0.93D and the relevant restriction sites are bracketed. The 74-base-pair (bp) sequence shown to contain the GS17 enhancer is marked by a double underline.

Promoter. The functional effect of an enhancer on a promoter is relatively independent of distance and orientation and, furthermore, an enhancer can stimulate the transcription of a heterologous promoter (for reviews, see refs. 23–25). To test the upstream region of the GS17 gene for enhancer activity, we have used the *Xenopus* major adult β-globin gene as a marker gene. The plasmid pXβ contains the entire β-globin gene, including about 500 bases of 5′ flanking sequence. Our previous experiments (7) and those of others (26) have shown that when this gene is injected into fertilized *Xenopus* eggs, transcription from the β-globin promoter occurs at a very low level during early development. In practice, transcripts are not observed under normal circumstances and are only detected at all when using very high specific activity probe and very long autoradiographic exposures. If the GS17 gene does indeed contain an enhancer we expect to see a significant increase in transcription at the MBT from the normally inactive β-globin promoter when the appropriate GS17 sequences are inserted next to the β-globin gene.

Approximately 350 bp of GS17 DNA, extending from the *Eco*RV site at −605 up to the endpoint of p0.93D (base −929), was tested for enhancer activity by inserting it into pXβ. The fragment was inserted, in both orientations, at the *Eco*RI site of pXβ, which is located 471 bases upstream of the β-globin cap site (see Fig. 3*A*). These constructions and unmodified pXβ were injected into fertilized *Xenopus* eggs and total RNA was isolated from groups of embryos at various times during subsequent development. The results of RNase protection experiments assaying for β-globin transcripts in

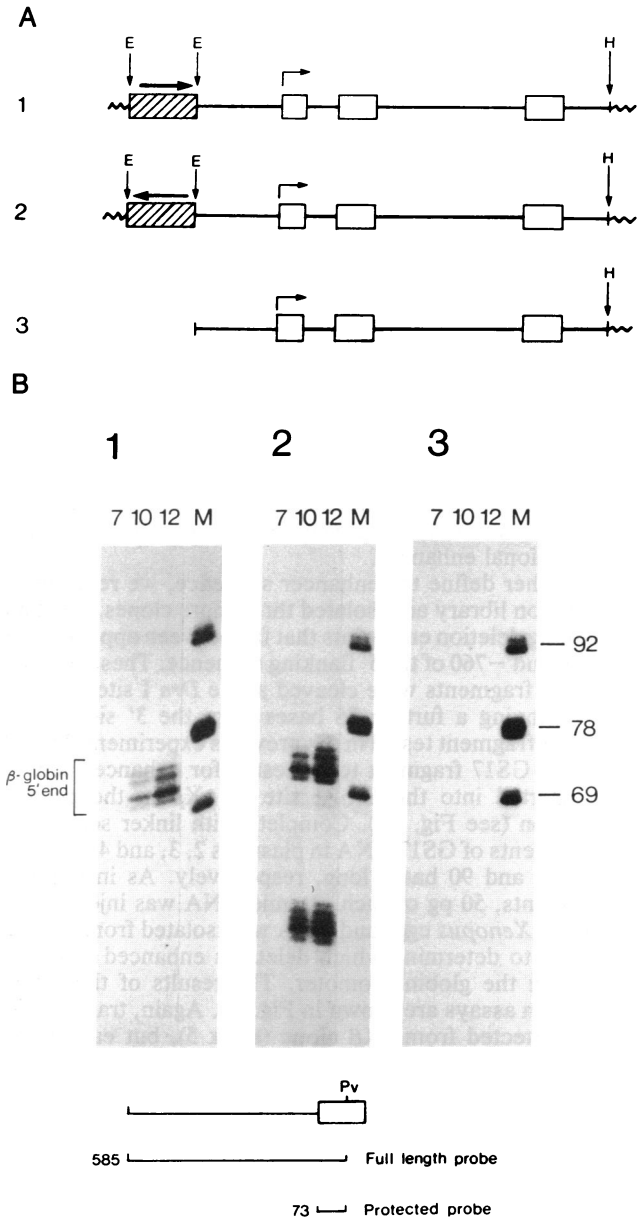

FIG. 3. Effect of GS17 gene sequences on the expression of the *Xenopus* β-globin gene in injected embryos. (*A*) Open boxes represent the exons and solid black lines represent the introns and flanking regions of the *Xenopus* β-globin gene. The 350-bp sequence from the 5′ flanking region of GS17 is a hatched box. Arrows above the box indicate the normal orientation of the fragment. pSP64 plasmid sequences are shown as ∿. (*B*) Fifty picograms of supercoiled DNA was injected into fertilized eggs as the first cleavage began. Injected eggs were allowed to develop to the stage indicated at the top of each track and total RNA was extracted. Transcripts from the β-globin gene were detected by RNase mapping using an SP6 RNA probe. RNA from one embryo equivalent was assayed and the protected fragments were fractionated on a 6% acrylamide/8.3 M urea gel. The β-globin-specific probe and the RNase-protected fragments that map the 5′ end of the β-globin gene are shown below the autoradiogram. Size markers (M) are *Hpa* II fragments of pBR322.

developing embryos are shown in Fig. 3*B*. As expected, no β-globin transcripts are detected in RNA isolated from embryos injected with unmodified pXβ. However, the presence of GS17 sequences in pXβ, in either orientation, causes a substantial increase in the amount of transcription from the β-globin promoter. Globin transcripts are absent from stage 7 RNA (pre-MBT) but are clearly visible in RNA extracted

from stage 10 embryos (post-MBT). The levels of globin RNA have increased further by stage 12 (corresponding to midgastrula), which was the last time point examined in this experiment. We do not know why two size classes of RNA are detected by the β-globin 5′ probe when the GS17 sequences are inserted into pXβ in the inverted orientation. These new protected fragments may be due to initiation of β-globin transcription at a second cap site. Alternatively, they could result from a transcript initiated within the inverted GS17 sequences and spliced to globin sequences. In the latter case, the GS17 sequences might act as an enhancer and a promoter (for example, see ref. 27).

These experiments show that a GS17 DNA fragment is capable of greatly increasing transcription from a heterologous promoter and that this GS17 sequence is active in both orientations. In separate experiments, the 350-base fragment was able to activate transcription from the GS17 promoter when placed about 1000 bases 3′ to the cap site of the gene (data not shown). We conclude therefore that sequences of the GS17 gene located between −605 and −929 contain a transcriptional enhancer.

To further define the enhancer sequence, we returned to the deletion library and isolated three more clones, this time containing deletion end points that fall between approximately −930 and −760 of the 5′ flanking sequence. These putative enhancer fragments were cleaved at the *Dra* I site at −681, thus removing a further 76 bases from the 3′ side of the enhancer fragment tested in the previous experiment. In each case, the GS17 fragment to be tested for enhancer activity was inserted into the *Eco*RI site of pXβ in the positive orientation (see Fig. 4*A*). Complete with linker sequences, the fragments of GS17 DNA in plasmids 2, 3, and 4 are about 200, 150, and 90 bases long, respectively. As in previous experiments, 50 pg of each plasmid DNA was injected into fertilized *Xenopus* eggs and RNA was isolated from stage 10 embryos to determine which deletions enhanced transcription from the globin promoter. The results of the RNase protection assays are shown in Fig. 4*B*. Again, transcription is not detected from pXβ alone (track 5), but each of the deletion constructions containing GS17 DNA significantly enhances β-globin transcription. The levels of β-globin transcript are about equal for each construction, suggesting that the entire enhancer domain is contained within the shortest fragment of GS17 DNA.

DNA sequencing shows that this short GS17 fragment contains 74 bases of GS17 DNA located between bases −681 and −755 of the 5′ flanking sequence. A number of other enhancer domains—for example, those associated with the human β-interferon gene (28), the human α-interferon gene (29), *Xenopus* ribosomal genes (30, 31), and mouse α-fetoprotein genes (32)—contain repeated elements and in some cases, maximum transcriptional activity is only obtained when all of the repeated elements are present. The 74-base region of GS17 does not contain any long direct or inverted repeats and sequences within this 74-base domain are not homologous to other sequences in the 5′ flanking region of the GS17 gene. We do note that the sequence CCCCT appears four times within the 74-bp region and this high GC content is reminiscent of the CCGCCC sequence found in the simian virus 40 enhancer. Comparison of the GS17 enhancer with the transcriptional control regions identified for *Xenopus* U1 and U2 small nuclear RNA genes (33, 34) does not reveal any striking homology. This is perhaps surprising because these small nuclear RNA genes are transcriptionally activated at the MBT (35).

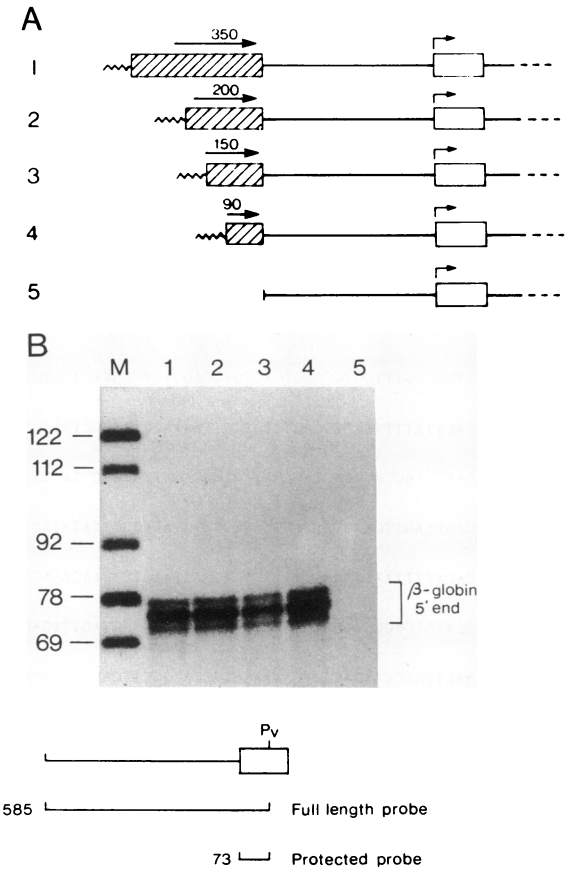

FIG. 4. Deletion analysis of the GS17 enhancer. (*A*) The open box represents the first exon of the *Xenopus* β-globin gene. The solid black line represents the 5′ flanking region and first intron of the *Xenopus* β-globin gene. Hatched boxes are fragments of the GS17 5′ flanking sequence and the number above each box is the approximate length of the fragment in bp (including *Eco*RI linker sequences). Arrows indicate the orientation of the fragment. (*B*) DNA representing each of the constructions shown in *A* was injected into fertilized eggs at the first cleavage. Embryos were allowed to develop until early gastrula, stage 10, and total RNA was extracted. Transcripts from the β-globin gene were detected by RNase mapping. Numbers above each track correspond to the DNA constructions, shown in *A*, that have been injected. Size markers (M) are *Hpa* II fragments of pBR322.

DISCUSSION

The aim of our experiments was to investigate the transcriptional regulation of genes expressed early in *Xenopus* embryogenesis and, in particular, to determine how some genes, but not others, are selected for transcription at the MBT. In this report we have identified at least one element of this regulatory machinery—namely, a cis-acting element located about 700 bases upstream of the GS17 promoter that has all of the properties of a typical transcriptional enhancer.

GS17 sequences are expressed for only a few hours during early embryogenesis. Transcription of the gene commences at the MBT and stops at about midgastrula, after which time the GS17 mRNA rapidly disappears (7). Using fertilized *Xenopus* eggs as an expression system we showed that injected copies of the GS17 gene exhibit a transcription profile identical to that of the endogenous gene. Experiments described in this paper delineate the regulatory sequences required for this transcriptional activation GS17. Deletion of sequences greater than about 800 bases upstream of the GS17 promoter has no noticeable effect on transcription of the injected gene, but deletion of sequences that contain the enhancer element causes transcription to drop below detectable levels. Furthermore, we have shown that insertion of the GS17 enhancer into a plasmid construction can cause heterologous genes to be efficiently transcribed during early

development. Placing the GS17 enhancer next to the *Xenopus* β-globin gene results in abundant transcription from the β-globin promoter, commencing at the MBT. Our analysis of the GS17 promoter has not been exhaustive and it is possible that other regulatory elements are present in the 5′ flanking region of the GS17 gene. Nonetheless, our results show that an enhancer plays an important role in regulating the transcription of GS17 sequences at the MBT.

The GS17 enhancer could mediate its effect as a site for some DNA modifications—e.g., methylation—but increasing evidence suggests that enhancers are binding sites for trans-acting cellular factors. For example, soluble factors bind to enhancers for an immunoglobulin heavy chain gene (36, 37), an insulin gene (38), and *Xenopus* ribosomal genes (31) and to enhancers of several viruses, including simian virus 40 (39, 40), polyoma (40), and murine sarcoma virus (39). If a trans-acting factor does indeed mediate transcription of the GS17 gene, several mechanisms for regulation can be suggested. For example, a trans-acting factor could be present in cleavage-stage embryos, perhaps bound to the GS17 enhancer sequence, but a general repressor of transcription such as that proposed by Newport and Kirschner (5) would prevent the expression of GS17 and other genes until the MBT. Alternatively, the factor might only be synthesized (or activated) at the MBT, leading to an immediate activation of the GS17 promoter. In either case, a trans-acting factor interacting with the GS17 gene is of particular interest because it must be of maternal origin. The absence of embryonic transcription prior to the MBT requires that the factor be stored as a maternal protein or as a maternal mRNA. Although our experiments have not investigated whether the enhancer is involved in the turn-off of GS17 transcription at midgastrula, it is plausible that the switch-on and switch-off of GS17 transcription is brought about by the appearance and disappearance of a regulatory protein that interacts with the enhancer.

Further experiments directed toward the identification and characterization of factors that bind to the GS17 enhancer sequence are necessary. Specifically, the presence of a trans-acting factor in the embryo may provide insight into the mechanism of regulation of GS17 expression. Recently, it has been shown that correct tissue-specific expression is obtained when *Xenopus* actin genes are injected into developing eggs (41, 42). Thus, it may soon be possible to compare the transcriptional regulation of GS17 with actin and other genes that are expressed early in *Xenopus* development.

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