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Differential Expression of the *Xenopus laevis* Tadpole and Adult β-Globin Genes When Injected into Fertilized *Xenopus laevis* Eggs

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Xenopus laevis tadpole and adult β-globin genes were injected into fertilized *X. laevis* eggs. Both injected genes replicated and were retained in the developing embryos with equal efficiency. Transcripts of the injected adult gene were detectable at gastrulation and reached a maximum level shortly thereafter. In contrast, transcripts of the injected tadpole gene were not detected until much later stages of development. The level of expression of both the injected genes was low compared with the level of expression of the chromosomal genes during erythropoiesis.

The transition from tadpole to adult globin gene expression in *Xenopus laevis* occurs at metamorphosis, the four electrophoretically resolvable tadpole polypeptides being replaced by six distinct adult proteins (6). The most abundantly expressed adult β-globin gene (β1) and the most abundantly expressed tadpole β-globin gene (βT1) have both been isolated by molecular cloning (9; D. Banville and J. G. Williams, manuscript in preparation). The βT1-globin gene is situated 22 kilobase pairs downstream from the β1-globin gene in the major globin locus (7; Banville and Williams, in preparation). Transcripts of the βT1-globin gene and two other tadpole β-globin genes are first detected by tail-bud stage, stages 28 to 30 (staging according to Nieuwkoop and Faber [8]). By stage 40, however, transcripts from the βT1-globin gene are present in vast excess over transcripts from the minor tadpole β-globin genes (Banville and Williams, in preparation).

Several studies have demonstrated the feasibility of injecting cloned genes into fertilized *X. laevis* eggs and analyzing their expression during development (1, 2, 10). In most of these experiments, circular, super-coiled DNA was injected. The DNA first replicated in this form to reach a maximum concentration during gastrulation. As development progressed, an increasing proportion of the DNA was found in a high-molecular-weight form comigrating with chromosomal DNA. Although there is no direct evidence for chromosomal integration, restriction enzyme digests of DNA from injected embryos yield fragments thought to contain junctions of injected and chromosomal DNAs (5). We have previously shown that the *X. laevis* adult αl- and β1-globin genes do not become detectably methylated after injection into *X. laevis* eggs and that both genes are expressed at low levels from their correct promoters with maximal expression near gastrulation (2). Similar results have been obtained by using sea urchin histone genes (1) and a rabbit β-globin gene (10). Taken together these results seem to imply that at least some cells at these early stages of development are permissive for the expression of any gene injected. This, however, does not seem to be the case for the tadpole βT1-globin gene since we show here that transcripts of the injected gene were not detected during early development. Only at relatively late stages of tadpole development was a low and variable level of expression of the injected βT1-globin gene found.

To distinguish transcripts of the injected genes from those of the endogenous genes, 12-nucleotide *Bam*HI linkers were inserted into the first exon of the major tadpole and adult β-globin genes (Fig. 1). The insertions were placed in the coding regions at sites not known to be involved in transcription or RNA processing. Also, the content and length of the insertions were selected so as not to generate nonsense codons and thereby interfere with translation and message stability. Transcripts of these insertion-modified genes were detected and distinguished from transcripts of the endogenous genes by primer extension analysis, using single-stranded DNA primers derived from sequences downstream of the insertions (2; Banville and Williams, in preparation).

The results presented in Fig. 2 are typical of a series of experiments analyzing expression of injected tadpole and adult β-globin genes during early development. Fertilized eggs were coinjected with equal amounts of pXGβT1(m) and pXGα1β1(m) DNAs. Samples were taken at several stages of early development and analyzed by Southern transfer for the presence of each of the injected DNAs (data not shown). Both DNAs behaved identically, showing the typical pattern of replication and persistence previously observed (2). By gastrulation, there was a 50- to 100-fold amplification of each input DNA, followed by a gradual loss during subsequent development. Transcripts from the modified adult β-globin gene were first detected in gastrulae (stage 11), were maximal before tail bud formation (stage 22), and continued to be detected in feeding tadpoles (stages 43 to 45) (Fig. 2, lanes 2 through 4). This is as expected from our experiments with the wild-type adult β-globin gene (2). The surprising result was that transcripts from the modified tadpole β-globin gene were not detected at any of the above stages (Fig. 2, lanes 13 through 15). The inability to detect transcripts of the injected tadpole βT1-globin gene during early development is not due merely to the relative sensitivities of the primer extension assays. We have performed control primer extension reactions using known amounts of tadpole or adult erythrocyte RNA, and we estimate that when transcription of the injected modified adult β-globin gene is maximal (stage 22), there are ca. 170 pg of modified adult β-globin gene transcripts per embryo and less than 6 pg of modified tadpole β-globin gene transcripts per embryo.

To establish that the 12-nucleotide insertion in the tadpole

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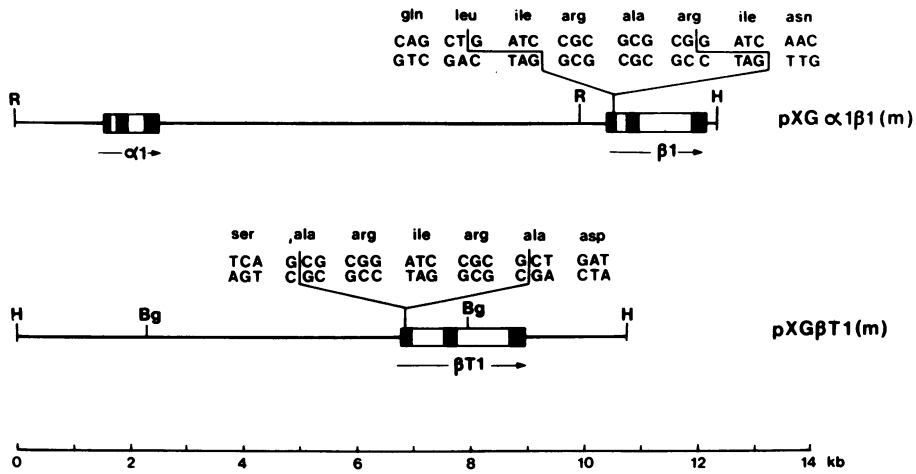

FIG. 1. Restriction maps of *X. laevis* genomic fragments cloned into pAT153 vectors (12). Clone pXGα1β1(m) was derived from λXGαβ103 (9) and contains the adult α1- and β1-globin genes. Using *Bam*HI linkers, we made a 12-base-pair insertion at the *Bcl*I site in the first exon of the β1-globin gene. Twelve-nucleotide *Bam*HI linkers were self-ligated, cleaved with *Bam*HI, and ligated via cohesive ends to *Bcl*I-cleaved β1-globin gene DNA. Clone pXGβT1(m) was derived from λXGβT106 (9; Banville and Williams, in preparation) and contains the tadpole βT1-globin gene. A 12-base-pair insertion was made at the *Pvu*II site in the first exon of the gene by blunt end ligating 12-nucleotide *Bam*HI linkers to *Pvu*II-cleaved βT1-globin gene DNA. The effects of the insertions on the DNA and protein sequences are shown above each β-globin gene. Each gene is represented by a box shaded for exons and unshaded for introns. Arrows indicate the direction of transcription. Restriction sites are labeled by letters: Bg (*Bgl*II), H (*Hin*dIII), and R (*Eco*RI).

β-globin gene was not somehow interfering with transcription, we coinjected eggs with the wild-type and insertion-modified tadpole βT1-globin genes. Transcription of the chromosomal tadpole β-globin genes is not detectable until tail bud formation (stages 28 to 30) (Banville and Williams, in preparation). Thus, in buffer-injected control samples taken at gastrulation, there were no tadpole β-globin gene transcripts (Fig. 2, lane 7). In coinjected samples taken at gastrulation, there were no tadpole β-globin gene transcripts, indicating that neither of the injected tadpole βT1-globin genes (wild type or insertion modified) was being transcribed (Fig. 2, lane 9). In coinjected samples taken at the feeding tadpole stage, there were wild-type transcripts (Fig. 2, lane 10), but these derived from the chromosomal gene as they were also present in the uninjected control sample taken at this stage (Fig. 2, lane 8). The samples were further analyzed by Southern transfer, and the results show that both injected DNAs replicated and persisted equally well (data not shown). Thus the 12-nucleotide insertion in the tadpole βT1-globin gene did not detectably alter replication, persistence, or expression of the gene.

Further experiments showed that the modified tadpole βT1-globin gene can be correctly transcribed after injection into fertilized eggs but only at relatively late stages of tadpole development. Three sets of fertilized eggs were injected with pXGβT1(m) plasmid DNA in injection buffer (1), and one set was injected with buffer alone (Fig. 3). Samples taken at both early and late stages of development were analyzed for transcripts of the modified tadpole βT1-globin gene. As seen previously in the coinjection experiments (Fig. 2), no transcripts of the insertion-modified tadpole βT1-globin gene were detected during the earlier stages of development (stages 1 to 45) (data not shown). In samples taken approximately 5 weeks later (stages 48 to 52), however, transcripts of the modified tadpole βT1-globin gene were present at detectable levels. Samples of RNA from the later stages were first analyzed by the primer extension assay. As expected, both buffer-injected and DNA-injected samples gave strong bands of the sizes expected from transcripts of the endogenous wild-type tadpole βT1-globin gene (Fig. 3a, lanes 5 through 8). In addition to wild-type transcripts, in one of the DNA-injected samples, there was a distinct band of the size predicted for transcripts of the injected insertion-modified βT1-globin gene (Fig. 3a, lane 8).

To confirm the results of this primer extension analysis, RNA from the late stage samples was also analyzed by S1 nuclease mapping. In this assay, the probe was a 100-nucleotide single-stranded DNA fragment, end labeled with ^{32}P at the *Bam*HI site that lies within the 12-base-pair insertion. The labeled end of this probe will hybridize only to transcripts of the insertion-modified gene, and thus the probe is totally specific for transcripts of the injected gene. By using this probe in S1 nuclease mapping assays, RNA from the buffer-injected sample gave no protected fragments (Fig. 3b, lane 1), whereas RNA from the three DNA-injected samples gave protected fragments of the sizes predicted for transcripts of the insertion-modified tadpole βT1-globin gene (Fig. 3b, lanes 2 through 4).

These results show that there are transcripts of the injected gene in all three DNA-injected samples, but that the sample which gave a positive result in primer extension analysis (Fig. 3a, lane 8, and Fig. 3b, lane 4) has a higher level of transcripts than the other two samples (Fig. 3a, lanes 6 and 7, and Fig. 3b, lanes 2 and 3). These differences in the amount of transcripts do not appear to be directly related to copy number of the injected gene. From DNA dot blots, we estimate that at this late stage the average number of copies of the injected gene per cell in the three samples was 0.3, 0.9, and 0.9, respectively (Fig. 3a, lanes 6 through 8, and Fig. 3b, lanes 2 through 4). Thus, it appears that the elevated level of transcripts in one of the samples results from a higher level of expression of the injected gene rather than a higher gene copy number. This variability in level of expression is presumably a reflection of variation in the fate of the injected DNA. Each tadpole is a complex mosaic with the injected DNA integrated into different cell types at various chromosomal sites (5). Comparison of the intensity of the primer

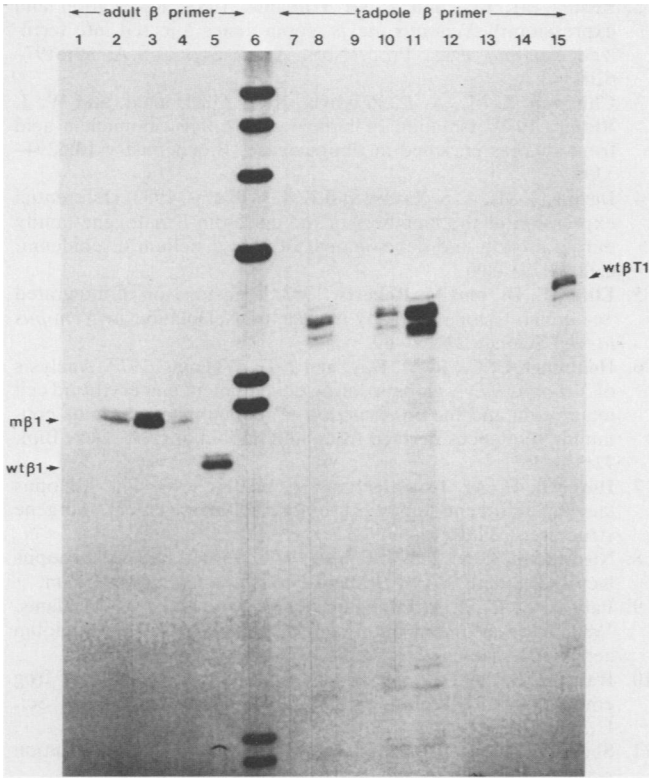

FIG. 2. The analysis of tadpole and adult β-globin gene transcription by primer extension. *X. laevis* eggs were collected, fertilized in vitro, chemically decapsulated, and injected before the first cleavage event with 50 to 100 pg of recombinant plasmid DNA (1). The eggs were allowed to develop normally with samples taken at relevant stages of development. Nucleic acids were prepared from samples taken at early stages by sodium dodecyl sulfate homogenization and phenol extraction (1). In the experiment presented, RNA from the equivalent of two eggs, embryos, or tadpoles was hybridized to either the tadpole or adult β-globin gene primer. Primer extension was as described elsewhere (2, 4; Banville and Williams, in preparation). Lanes 7 and 8 are from gastrulae (stage 11) and feeding tadpoles (stages 43 to 45), respectively, injected as eggs with buffer. Lanes 9 and 10 are from gastrulae and feeding tadpoles, respectively, coinjected as eggs with pXGβT1 and pXGβT1(m). Lanes 1 through 4 and 12 through 15 are from samples coinjected as eggs with pXGα1β1(m) and pXGβT1(m). Lanes 1 and 12 are from eggs (stage 1), lanes 2 and 13 are from gastrulae (stage 11), lanes 3 and 14 are from pretail bud (stage 22), and lanes 4 and 15 are from feeding tadpoles (stages 43 to 45). Lane 5 is a control with 300 pg of polyadenylated RNA from adult erythrocytes, and lane 11 is a control with total RNA from tadpoles at stages 40 to 45. Lane 6 is a DNA size marker, a *Hin*fI digest of pAT153. The positions of the extended fragments from wild-type (wtβ1) and insertion-modified (mβ1) adult β1-globin gene transcripts are indicated on the left. On the right the positions of the extended fragments from wild-type tadpole βT1-globin gene transcripts (wtβT1) are indicated.

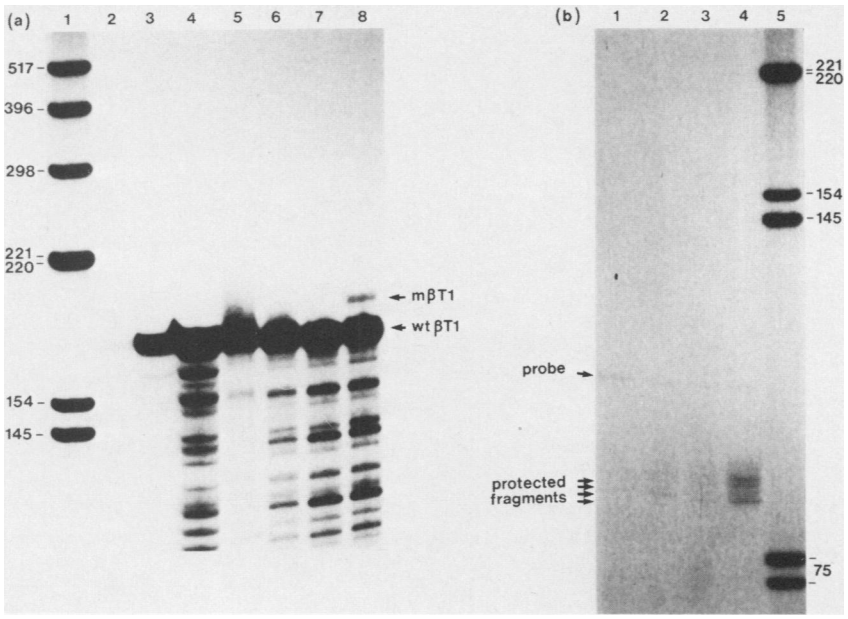

FIG. 3. Primer extension and S1 nuclease mapping analyses of RNA extracted from late stage tadpoles injected as eggs with pXGβT1(m) plasmid DNA. In samples taken at later stages, the tadpoles were cut in half longitudinally, and DNA was prepared from one half by sodium dodecyl sulfate homogenization and phenol extraction (1), whereas RNA was prepared from the other half by homogenization in 4 M guanidinium thiocyanate, followed by centrifugation through 5.7 M CsCl (3; Banville and Williams, in preparation). In panel a, 50-μg samples of total RNA from late stage tadpoles (stages 48 to 52) were analyzed by primer extension. Lane 5 is RNA from buffer-injected tadpoles. Lanes 6 through 8 are RNA from three different sets of DNA-injected tadpoles. Lanes 2 through 4 are controls with 0.1, 1.0, and 10 ng of polyadenylated RNA from late-stage tadpole erythrocytes. Lane 1 is a *Hin*fI digest of pAT153. In panel b, 50 μg of total RNA from the same late-stage samples was analyzed by S1 nuclease mapping. The labeled 100-nucleotide single-stranded probe described in the text was hybridized to the RNA samples as in the primer extension assays (4). Subsequent S1 nuclease digestion was as described (11), and the samples were analyzed on 10% acrylamide-urea gels. Transcripts of the insertion-modified tadpole βT1-globin gene were predicted to give protected fragments 82 to 85 nucleotides in length. Lane 2 is RNA from buffer-injected tadpoles. Lanes 2 through 4 are RNA from three different sets of DNA-injected tadpoles. Lane 5 is a *Hin*fI digest of pAT153.

extension products derived from transcripts of the injected gene with those corresponding to transcripts of the endogenous gene (Fig. 3a) indicates that transcripts of the injected gene are present at relatively low levels. The data clearly show, however, that in at least some tissues of some late-stage tadpoles the insertion-modified tadpole βT1-globin gene is transcribed at low levels but from the correct promoter.

We conclude that the lack of expression of the injected tadpole βT1-globin gene during early development is a consequence of some intrinsic difference between the tadpole βT1-globin gene and the adult β1-globin gene. This difference between the tadpole βT1- and adult β1-globin gene promoters is rather surprising since each gene is the most abundantly transcribed β-globin gene at its respective stages of maximal expression, and hence, both genes would be expected to have strong promoters. Perhaps even more puzzling is the fact that the injected adult gene is expressed during early tadpole stages, whereas the injected tadpole gene is not. This anomalous pattern of expression suggests that it may not be possible to use microinjection of cloned genes into *X. laevis* eggs to analyze developmental regulation of globin gene expression.

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