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Expression of adult and tadpole specific globin genes from *Xenopus laevis* in transgenic mice

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ABSTRACT

Transgenic mice were generated which carried the adult α and β-globin genes and the major tadpole specific β-globin gene of *Xenopus laevis*. The adult specific α and β genes were found to express in erythroid tissues in adult mice, while the major tadpole specific β gene (βT1) was expressed in blood from 12.5 day embryos. The pattern of expression of the βT1 gene during mouse development was consistent with its being regulated as an embryonic globin gene in the mouse. This observation suggests that some of the factors mediating globin switching have been conserved during the evolution of modern amphibia and mammals and raises interesting questions concerning the evolution of vertebrate globin gene switching.

INTRODUCTION

The globin chains of *Xenopus laevis* are encoded by several genes which are clustered together and subject to differential regulation during development, a situation which is similar to that found in mammals and birds (reviewed in ref. 1). However, a major difference between the organisation of the *Xenopus* globin genes and that of higher vertebrates is the close linkage of the *Xenopus* α and β genes. *Xenopus laevis* possesses two globin gene clusters, each containing tadpole and adult specific α and β-globin genes (2,3) (Fig. 1). A complete switch from production of tadpole α and β chains to production of the adult chains occurs during metamorphosis (4,5,6,7). Although many of the genes have been cloned and sequenced and a considerable amount of information has been gained on their expression during development, the lack of an expression system has prevented any direct analysis of the regulatory elements responsible for *Xenopus* globin gene regulation. It would be particularly interesting to know whether the *Xenopus* globin clusters contain discrete regulatory regions equivalent to the mammalian globin locus control regions (LCRs) (8) and, if so, where such regions are located. In an attempt to establish an expression system which would allow the study of *Xenopus* globin gene regulation, we have generated transgenic mice carrying the genes encoding the adult α and β chains and the major tadpole β chain. All three genes were found to show erythroid specific expression. We also present evidence that the major tadpole specific β-globin gene of *Xenopus laevis* is subject to differential regulation during mouse development, an observation which has interesting implications for the evolution of globin switching in vertebrates.

MATERIALS AND METHODS

Plasmids

The linked *Xenopus laevis* adult α and β globin genes are contained on the plasmid pXGαβ1.2 (9). The 15kb fragment containing the genes was released from vector sequences by digestion with EcoR1 (Fig. 1). A 10 kb Hind III fragment (Fig. 1) containing the major tadpole β-globin gene, βT1 (6,10) was purified from the plasmid pXGβt10.

Transgenic mice

DNA fragments were purified from vector sequences by agarose gel electrophoresis and injected into the pronuclei of mouse oocytes as previously described (11). Transgenic animals were identified by Southern blot analysis of tail DNA. Copy number was estimated by comparison with different loadings of plasmid DNA. Tissues for developmental analysis were obtained by mating founders to non-transgenic animals and dissecting embryos at the appropriate day of gestation (the day the mating plug was observed was designated day 0.5). Transgenic embryos were identified by Southern blotting of placental DNA. Adult mice were made anemic by three sequential subcutaneous injections of 0.4% phenylhydrazine (0.01ml/g body weight). Injections were carried out at 12 hour intervals and animals were sacrificed and tissues harvested on day 5 after the first injection.

Analysis of *Xenopus* and mouse globin gene expression in mouse tissues

RNA was purified from adult tissues (with the exception of blood) by homogenisation in 4M guanidinium thiocyanate, 0.1M β-mercaptoethanol followed by centrifugation through a 5M

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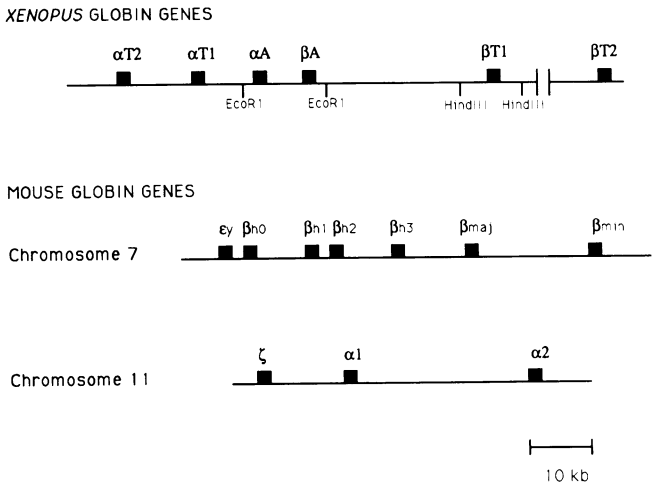

Fig. 1. Organisation of the α and β-globin genes of *Xenopus laevis* and of the mouse. The restriction enzyme sites shown in the *Xenopus* globin locus are those which were used to isolate fragments for micro-injection.

caesium chloride cushion (12). Embryonic tissues and blood from all stages were lysed in 6M guanidinium hydrochloride and extracted with phenol chloroform. Total nucleic acid was then precipitated with ethanol and used for primer extension analysis.

Primer extensions were carried out as described in ref. 13 using synthetic 25 base oligomers which were 5'end-labelled using T4 polynucleotide kinase. The sequences of the primers used were as follows.

Xenopus adult α-globin: 5'GCGATAGCAGGCATAATTGCC-TTGA 3'. Complementary sequence +73 to +98. Size of extension product: 98 bases. *Xenopus* adult β-globin: 5'GAT-CAGCTGACGATCATGTGCTGTC 3'. Complementary sequence +54 to +79. Size of extension product: 79 bases. Mouse β_{maj}: 5'AAGAGACAGCAGCCTTCTCAGCATC 3'. Complementary sequence +65 to +90. Size of extension product: 90 bases. *Xenopus* βT1: Two different primers were used. The extension shown in Fig. 2A was carried out with the primer 5'GGCGTTAATGGCGGATTTCTCATC 3', complementary to the sequence from +73 to +98 and giving an extension product of 98 bases. For the analysis shown in Fig. 2B, the sequence of the primer was 5'GACAGGTGC-ACCATGGTGGCTGTAG 3', complementary to the sequence from +55 to +70 and giving an extension product of 70 bases.

RESULTS

Expression of the adult and β genes

The adult α and β globin genes of *Xenopus laevis* were injected into mouse oocytes on a 15 kb fragment and transgenic pups were identified by Southern blotting of tail DNA. A total of eight animals were identified as carrying the intact transgene at varying copy number. Because levels of expression were expected to be low relative to the endogenous genes, adult transgenic animals were made anemic by injection with phenylhydrazine and RNA from blood, spleen and other tissues was analysed for the presence of *Xenopus* globin transcripts. One of the eight founder animals carrying five copies of the transgene, showed significant levels of transcripts from both the α and β genes in the blood and spleen and not in other tissues (Fig 2, panels A and B). The level of expression of the *Xenopus* genes was 1−2% of the β_{maj} gene

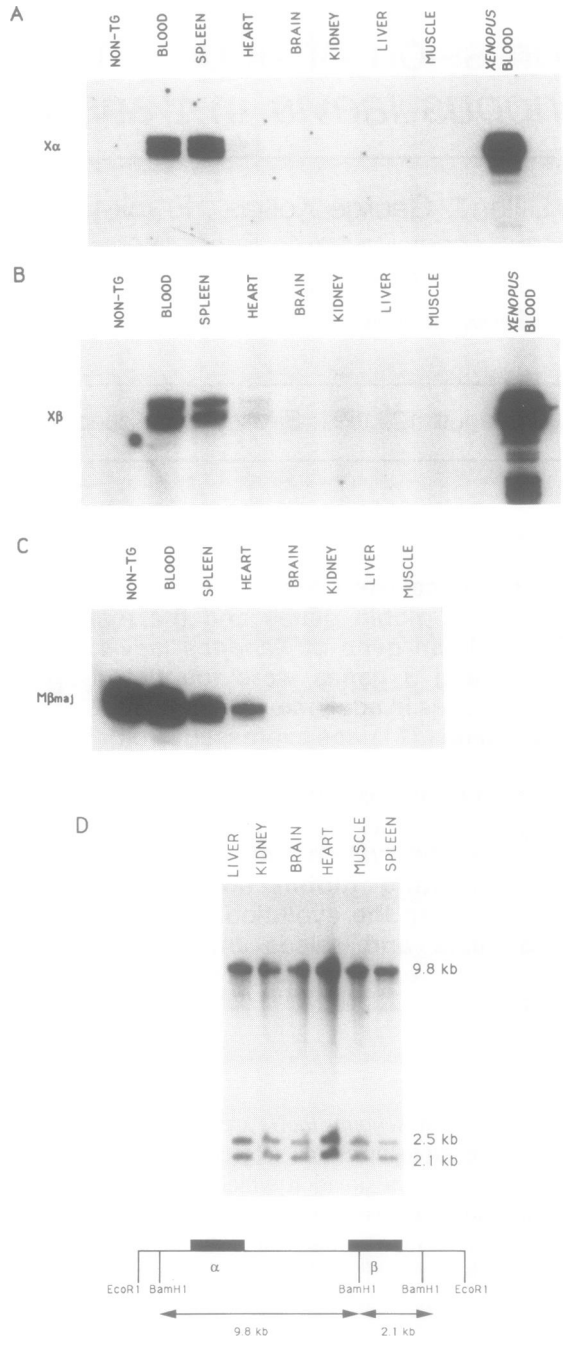

Fig. 2. Primer extension analysis of *Xenopus* and mouse globin RNAs in tissues from a transgenic mouse carrying the *Xenopus* α and β-globin genes. Primer extension was carried out on 20μg of RNA from each tissue using the oligonucleotides described in Materials and Methods labelled to equivalent specific activities. Control reactions were carried out on 100ng of anemic *Xenopus* blood. Non-TG denotes anemic non-transgenic mouse blood. Sizes of primer extension products are given in Materials and Methods and were confirmed by comparison with labelled markers (not shown). The exposure time for the mouse β_{maj} band was one tenth that of the *Xenopus* globin bands. The relative intensity of the *Xenopus* globin extension products compared with that for mouse β_{maj} were estimated by densitometric scanning of equivalent exposures. A. Analysis of *Xenopus* α-globin expression. B. Analysis of *Xenopus* β-globin expression. C. Analysis of expression of the mouse β_{maj}-globin gene. D. Southern blot analysis of DNA from the transgenic animal whose globin expression profile is shown in panels A,B and C. Equal amounts of DNA from each tissue were digested with BamH1 and and subjected to Southern blot analysis. The blot was probed with the entire 15 kb EcoR1 fragment used to make the transgenic mouse. The 9.8 kb and 2.1 kb bands come from internal fragments while the 2.5 kb band is a junction fragment resulting from tandem end to end joining of two copies of the injected fragment.

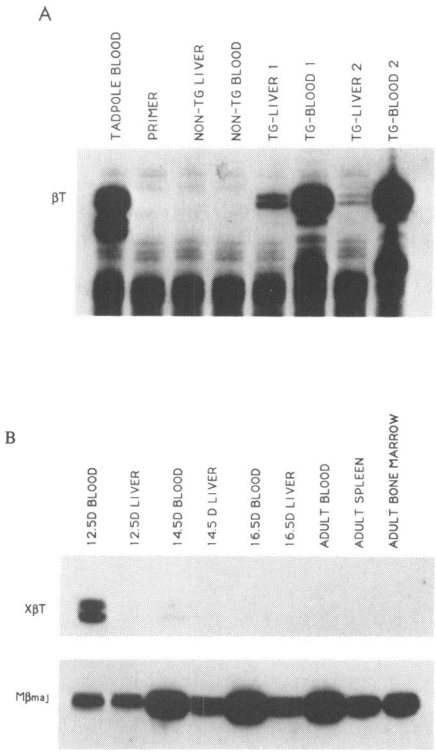

Fig. 3. Primer extension analysis of transcripts from the *Xenopus* βT1 gene at different stages of mouse development. A. Analysis of βT1 expression in liver and blood from two transgenic (TG) and one non-transgenic (non-TG) fetus at 12.5 days gestation. Each reaction contained 4μg of RNA. The control reaction was carried out on 100ng of tadpole blood. The additional lower mobility band observed for tadpole blood is likely to result from a closely related tadpole β-globin mRNA which cross-reacts with the primer (see ref. 7). B. Relative levels of expression of the *Xenopus* βT1 and mouse β_{maj} genes in liver and blood from fetuses at different stages of gestation, in adult anemic blood and spleen and in non-anemic adult bone-marrow. Primer extension was carried out on 4μg of RNA from each stage with the Xenopus βT1 primer and 100ng of RNA with the mouse β_{maj} primer.

which is similar to that which has been observed for the human β-globin gene without the LCR in transgenic mice (11). The expressing founder animal failed to pass the transgene to its progeny, showing that it was mosaic in the germline. In order to check whether this mosaicism extended to other tissues, DNA from the tissues which were analysed for *Xenopus* globin expression was probed for the presence of the transgenes by Southern blotting (Fig 2D). DNA from spleen, which expressed the *Xenopus* globin genes, gave a signal for the transgenes which was equivalent to that of liver, kidney, brain and muscle, which did not show expression. Although DNA from the heart gave a 2-fold stronger signal on Southern blotting than the other tissues suggesting that there may be some somatic mosaicism in this animal, the relative level of *Xenopus* globin expression in heart was consistent with that observed for mouse β_{maj} indicating it was due to contaminating blood. Therefore, we conclude that the pattern of *Xenopus* globin gene expression in this animal is genuinely tissue specific. A second line of animals also showed detectable expression of the transgenes in the blood and spleen, but at levels which were approximately 20-fold less than those observed in the animal described above (data not shown). The lower level of expression is not related to copy number as this line had a higher copy number (8 copies) than the expressing animal.

Expression of the βT1 gene

As a further test of the ability of mouse erythroid factors to recognise *Xenopus laevis* globin promoters, we generated transgenic mice carrying the major *Xenopus* tadpole specific globin gene βT1 (6,10). The βT1 gene was injected as a 10 kb Hind III fragment and two transgenic lines were analysed for expression of the transgene at different stages of development. In one of these lines, expression of the tadpole globin gene was detected in liver and blood from fetuses at 12.5 days gestation (Fig. 3A). The level of expression was much higher in blood than in liver, a pattern which is consistent with embryonic erythroid expression. Transcripts from the βT1 transgene were also detected in RNA from 10.5 day yolk sac at a level of 1−2% of that of the mouse βh1 gene (data not shown). No expression was detected in the other line indicating that expression of the transgene is position sensitive.

Developmental regulation of the βT1 gene

In order to establish whether the βT1 gene is subject to developmental regulation in the mouse, the level of βT1 transcripts was analysed in RNA from liver and blood from different stages of fetal development in the expressing line as well as from adult anemic spleen and blood and non-anemic bone marrow (Fig. 3B). Expression was found to decrease sharply from 12.5 days onwards and was reduced by greater than 100-fold in adult erythroid tissues. Analysis of mouse β_{maj} transcripts in the same samples showed the expected increase in expression over the same period. The pattern of expression in fetal blood and liver is again exactly that which would be expected for an embryonic globin gene (14,15).

DISCUSSION

Our results demonstrate that the globin genes of *Xenopus laevis* can be expressed in a tissue specific manner in transgenic mice. Since the divergence of the vertebrate lineages which ultimately gave rise to modern amphibia and mammals is thought to have taken place around 350 million years ago (16), this indicates a high degree of conservation of the molecular interactions which control the regulation of the vertebrate globin genes. Attempts to express the *Xenopus* globin genes transiently in developing tadpoles following injection of the fertilized egg have not been successful (17 and N.D. unpublished) and it has not proved feasible to construct lines of transgenic *Xenopus laevis* because of their very long generation time (two years). The finding that the genes are expressed in transgenic mice provides an alternative system by which the regulatory sequences of this interesting gene cluster could be characterised. In this study, we have found that the level of expression of the *Xenopus* genes in transgenic mice is much lower than that of the endogenous mouse genes. The failure of the genes to express in many of the lines analysed also indicates that expression is highly position sensitive. Although we cannot be certain that LCR sequences from *Xenopus* (if they exist) would be fully recognised by murine factors, sequences from the chicken β globin locus have been found to be capable of conferring position insensitive copy-dependent expression on the chicken β-globin gene in transgenic mice (18). This suggests that the *Xenopus* globin LCR is not located within the sequences used in this study.

We have also found that a tadpole specific β-globin gene from *Xenopus laevis* is apparently subject to developmental regulation in erythroid tissues in the single transgenic mouse line which we

have studied. Although this result is derived from a single mouse line, the expression pattern is precisely that which would be expected for a mouse embryonic globin gene making it very unlikely that it results from a position effect. Existing theories concerning the evolution of vertebrate globin genes specific for different developmental stages rest entirely on comparative sequence analysis (reviewed in ref. 19). Such analysis has indicated that the embryonic, fetal and adult β-globin genes of modern mammals are the result of relatively recent duplication events which occurred after the divergence of mammalian and avian lineages. This has led to the supposition that globin gene switching evolved independently in the different vertebrate orders. However, there are other considerations which suggest that comparative sequence analysis may show only part of a more complicated picture. Multiple globin genes are found in all vertebrate orders, including fish, and the vital role they play in allowing the organism to adapt to different respiratory environments suggests that they are likely to have arisen very early in vertebrate evolution. Sequence analysis has indicated that the duplication event which gave rise to the *Xenopus laevis* βT1 gene was indeed a very ancient event (20,21) and analysis of globin chain sequences from Coelocanth fish has shown that the Coelocanth globin chains are more closely related to amphibian tadpole globins than to any other vertebrate globins including those of Teleost fish (22).

The more recent origin of the mammalian embryonic and fetal genes suggests that these genes may have superseded a more ancient set of differentially regulated globin genes. The organisation of modern mammalian globin clusters with a discrete locus control region located at the boundary of the gene cluster suggests a situation where there might be a considerable degree of flexibility in reprogramming the expression pattern of newly duplicated genes through gene conversions and large deletions, giving rise to fusion events altering the control elements surrounding genes. Events of this type have been shown to occur frequently in the modern mammalian β-globin loci (19). Comparative analysis of the regulatory sequences which control the globin genes of different vertebrate orders could provide a valuable addition to sequence comparison in attempting to understand the role of such events in the evolution of the globin gene clusters. The expression system which we describe in this paper provides one means for carrying out such analysis.

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