The organization of the tadpole and adult α globin genes of *Xenopus laevis*

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ABSTRACT
     Adult erythrocytes of X. laevis contain six electrophoretically resolv-
able globin polypeptides while tadpole erythrocytes contain four polypep-
tides, none of which comigrates with an adult protein. We show that three
of the adult proteins are α globin polypeptides (α1, α2, α3) and three are
β globin polypeptides (β1, β2, β3). We find that a tadpole α globin gene
(αT1) is linked to the major adult locus in the sequence 5'-αT1-α1-β1-3'
with 5.2 kb separating αT1 from α1.   Another tadpole α globin gene (αT2)
is linked to the minor adult locus in the sequence 5'-αT2-α2-β2-3' with
10.7 kb separating αT2 from α2. These linkage relationships are consistent
with the major and minor loci having arisen by tetraploidization but the
different separation of larval and adult globin genes at the two loci
indicates the occurrence of some additional chromosomal rearrangement.
Two alternative models are presented.

INTRODUCTION

     Vertebrate α and β globin genes are thought to have arisen as the

result of the duplication, and subsequent divergence, of an ancestral

globin gene some 500 My ago (1). In X. laevis, adult α and β globin

genes are linked in the genome (2,3) suggesting that in amphibia the genes

have retained their original relative location.

     X. laevis has nearly twice the C-value and twice the chromosome number

of another Xenopus species, X. tropicalis (4,5). Using X. laevis α and

β globin cDNA clones as hybridization probes, X. tropicalis was shown to

possess a single locus containing an α globin gene linked to a β globin

gene (2). In X. laevis two loci were detected. One of the loci contains

the genes encoding the major adult α and β globin polypeptides (α1 and

β1). This locus was cloned in a λ vector and the internal organization

of the α1 and β1 genes was determined (3). The other locus detected by

Southern mapping contained a similarly linked pair of genes termed α2 and

β2. There was no evidence for linkage of the α1-β1 locus to the α2-β2

locus. These various pieces of evidence led to the suggestion that the

two loci arose as the result of whole chromosome duplication during tetra-
ploidization (2).

Adult erythrocytes from X. laevis contain six electrophoretically
resolvable globin polypeptides (6). We describe here the isolation of a
genomic clone containing the α2 globin gene and we show that it encodes
one of the minor globin polypeptides which we term α2. We have also
isolated an adult α globin gene for which no linkage data is as yet avail-
able and we show that it encodes another of the minor globin polypeptides
which we term α3.

Erythrocytes isolated from tadpoles of X. laevis contain four electro-
phoretically resolvable globin polypeptides, none of which co-migrates with
an adult globin gene protein (6). We have isolated and characterized a
number of λ genomic clones containing tadpole globin genes and we find that
the α1-β1 locus and the α2-β2 locus are each linked to a tadpole α globin
gene in the order (5'-αT-α-β-3'). However, the αT and α genes are separated
by different distances at the two loci, and this suggests two models for
tadpole globin gene evolution.

MATERIALS AND METHODS
Construction and screening of clone banks

We have described previously the preparation of a Charon 4 clone bank
containing X. laevis DNA partially-digested with Hae III and Alu I (3).
The procedure for screening the bank with adult globin cDNA and rescreening
positives with the α1 cDNA clone (pXG6C1) and the β1 cDNA clone (pXG8D2)
was also described.

The partial Eco R1 cosmid library was constructed using DNA from the
same individual X. laevis as the Charon 4 bank. Fragments of > 20 kb in
size were ligated into Eco R1-cut Homer I cosmid vector (7). Recombinants
were packaged in vitro using the method of Sternberg et al., (8), as
modified by Scherer et al., (9), using strains BHB2688 and BHB2690 (10).
Packaged phage were grown in a temperature-sensitive lysogen of E. coli
to facilitate screening (11). Lysed colonies (3.5×10^5) were pooled and
then 10^6 colonies were plated out for screening twice with adult globin
^{32}P-cDNA. Cosmid DNA from positive colonies was isolated by a 'mini-
preparation' procedure (12), digested by Eco R1, electrophoresed in
agarose, transferred to nitrocellulose filters and probed with adult
globin cDNA, tadpole globin cDNA, pXG6C1 and pXG8D2. Two identical
colonies were isolated each containing a 14.3 kb fragment which hybridized

to adult globin cDNA, pXG6C1 and pXG8D2. This clone is called cXGαβ4.2.

Dot Blot Hybridizations and Southern Transfers

Dot blot hybridizations (13) were carried out essentially as described by Thomas (14). Cloned DNA (1μg) was denatured in 0.3 M NaOH at room temperature for 15 min, neutralized and samples (< 10 μl) were then spotted onto nitrocellulose filters which had been pre-soaked in 20XSSC and dried at room temperature. Gel electrophoresis and Southern transfers (15) were carried out as described previously (3). The dot blot and Southern filters were baked at 80° for >4 hr and hybridized with radioactive probes under conditions described by Maniatis et al., (16). Restriction fragment purification was effected by electro-elution from an agarose slice followed by elution through a DE52 column and ethanol precipitation. The fragment was then labelled by nick translation (17) for use as a probe.

Adult and Tadpole RNA and cDNA preparations

Blood was collected from X. laevis in heparinized Stearns' solution (18) by cardiac puncture for tadpoles and adults or alternatively, for tadpoles, by harvesting blood from animals in which the tail had been amputated (H. Woodland, personal communication). Isolation of RNA from these cells was carried out as described by Hentschel et al., (6) omitting size selection on a sucrose gradient. ^{32}P-cDNA was prepared from mRNA by the method of Williams and Penman (19) using AMV reverse transcriptase kindly supplied by J. Beard.

Hybrid-arrested cell-free translations

Recombinant phage DNA (20 μg) was hybridized to adult erythroblast cytoplasmic RNA (7 μg) essentially as described by Paterson et al., (20) except that the DNA was dissolved in formamide before the addition of hybridization buffer, and the cloned DNA was denatured by heating at 80° for 2 min before the RNA was added. After hybridization at 48° for 4 hr, half the sample was heated at 70° for 2 min and then ethanol precipitated, the other half was ethanol precipitated directly. These two samples are referred to as the 'boiled' and 'hybridized' samples. Cell-free translations were performed in the presence of ^{3}H-histidine in a wheat germ extract as described by Roberts and Paterson (21) with the modifications used previously (6). Acid-urea polyacrylamide gel electrophoresis was carried out as previously described (6) except that samples were loaded in 9.5 M urea in 5% acetic acid to prevent preferential precipitation of β-globin polypeptides.

DNA sequencing

DNA sequence was determined in both strands by the method of Maxam
and Gilbert (22) on thin (0.3 mm) polyacrylamide gels. Restriction frag-
ments were labelled at their 5'termini using calf intestinal phosphatase
(Böehringer, 23) and T4 polynucleotide kinase (PL, 22). 3'labelling was
carried out using AMV reverse transcriptase (J. Beard) as described by
Williams and Penman (19), except that actinomycin D was omitted.

RESULTS

1.   Isolation and characterization of the genes encoding the two minor
      adult α-globin polypeptides

      We have previously described the construction of two bacteriophage
λ genomic clone banks containing fragments of X. laevis DNA and the isola-
tion from these banks of the major adult α- and β-globin genes, α1 and β1
(3). We have now isolated from the bank prepared by partial digestion with
Hae III + Alu I (16), a number of other genomic clones which hybridize to
adult globin cDNA. Two of these λ clones which we term λXGα101 and λXGα102
hybridize to the α1 cDNA clone pXG6C1 (24) but not to the β1 cDNA clone
pXG8D2 (24) indicating that they contain α globin genes.

      To determine which of the six polypeptides found in adult blood (6)
were encoded by these two genes, we performed hybrid-arrested translation
(HART) experiments (20). When λXGα102 is hybridized to adult globin mRNA
it prevents translation of the fastest-migrating polypeptide, while λXGα101
prevents translation of the third fastest-migrating polypeptide (Figure 1).
We have previously shown that the second fastest-migrating polypeptide is
the major adult α globin polypeptide which we term α1 (24). Thus the three
fastest-migrating polypeptides are all α-globins. This conclusion was con-
firmed by HART experiments with the cDNA clones pXG6C1 and pXG8D2 under
hybridization conditions of reduced stringency. Translation of the three
fastest-migrating polypeptides was prevented by the α globin cDNA clone
pXG6C1 and that of the three slowest-migrating polypeptides was prevented
by the β globin cDNA clone pXG8D2 (result not shown).

      Using the α1 and β1 globin cDNA clones as probes in Southern transfer
analysis of genomic DNA, restriction maps of two loci (α1-β1 and α2-β2)
were obtained (2). The α and β globin genes at the locus showing weaker
hybridization were assumed to encode minor α and β globin polypeptides and
were termed α2 and β2. In the region common to both, the restriction map
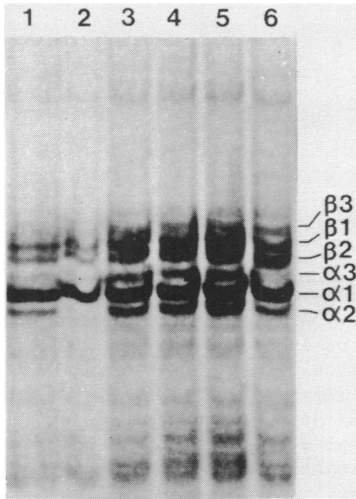of λXGα102 and the map of the α2-β2 locus (2) are almost identical (Figure

Fig. 1.   Hybrid-arrested translation experiments using adult erythroblast cytoplasmic RNA with λXGα102 (Lanes 1 and 2), a control λ clone containing no known globin gene (Lanes 3 and 4), and λXGα101 (Lanes 5 and 6). Lanes 2, 4 and 6 contain the hybridized samples, and Lanes 1, 3 and 5 contain the boiled samples. The identity of each polypeptide is indicated on the right.

2) and we conclude that λXGα102 contains the α2 gene. We therefore term the polypeptide encoded by λXGα102 the α2 polypeptide (Figure 1). We term the remaining polypeptide, that encoded by the gene in λXGα101, the α3 polypeptide (Figure 1).

    Translation of the third most slowly-migrating polypeptide can be prevented by hybridization with the cDNA clone pXG2β2 (6). This clone hybridized more strongly to genomic restriction fragments containing the β2-globin gene than to those containing β1, (A. Jeffreys, personal communication), we therefore term this the β2 polypeptide. We will call the remaining β-globin polypeptide, for which no cloned probe is as yet available, β3 (Figure 1).

2.   A tadpole α-globin gene (αT1) is linked to α1

    A genomic clone, which we term λXGαT105, which was isolated from the partial Hae III + Alu I bank because it hybridized with adult globin cDNA, also hybridized with tadpole globin cDNA. The restriction map of this clone was established and the nucleotide sequence of the restriction fragment which displayed most hybridization with tadpole globin cDNA was determined (Figure 3). The sequence contains an open reading frame which translates into 41 amino acids. All but 7 of these 41 amino acids are identical to amino acids found at the C-terminus of either the human α-globin or X. laevis adult α1 globin proteins (Figure 3). In addition, the sequence is very similar, but not identical (see legend to Figure 3), to the larval α1 partial sequence published by Widmer et al., (25). In our sequence
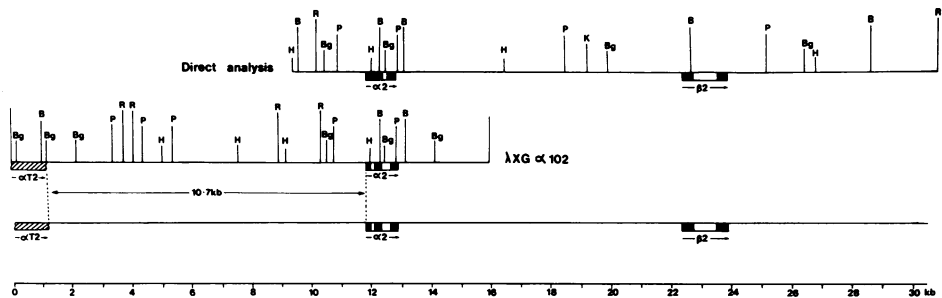
Fig. 2.   Restriction map of the minor globin locus in X. laevis.
Restriction sites are indicated by vertical lines and letters:
(B) Bam HI, (Bg) Bgl II, (H) Hind III, (K) Kpn I, (P) Pst I,
(R) Eco R1. The vertical lines at each end of λXGα102 represent
the Eco R1 sites constructed in the cloning procedure. The top
line is the map derived from Southern transfer analysis of total
genomic DNA, redrawn from Jeffreys et al., (2), the middle line
is the map of the insert in the Charon 4 recombinant, λXGα102.
These two maps have been aligned using the restriction sites
common to both. The complete locus containing the three globin
genes has been drawn below for clarity. All three lines were
drawn to the scale at the bottom of the figure. The positions of
the α and β globin genes are indicated by boxes below the line,
black for exons, white for introns and hatched for unknown, as
determined by: R-looping, Southern blotting and comparison with
α1 for α2; Southern blotting and comparison with β1 for β2 (2);
Southern blotting for αT2. A horizontal arrow denotes the
direction of transcription. This was determined for αT2 by using
as probes in Southern analysis of λXGα102, the Eco R1 fragment
containing the whole of exon 3 from αT1, including the 3' non-
coding sequences, and the Eco R1-Bgl II fragment from the same
region of αT1 containing the coding sequences of exon 3 only
(Figs. 3 and 4). The Eco R1 fragment hybridized to all the
fragments spanning the hatched region representing αT2 but the
Eco R1-Bgl II fragment hybridized only to the fragment left of
the Bgl II site at the extreme left of the insert. The restric-
tion map of αT2 shares no sites with that of the larval α2 of
Widmer et al., (25).

the presence of a consensus intron/exon signal strengthens the conclusion

that this sequence represents the third exon and part of the second intron

of an α globin gene. The gene will therefore be referred to as αT1 and the

clone containing it as λXGαT105. The adult globin gene contained within

this clone is the α1 globin gene (Figure 4). One of the clones (λXGαβ103)

which contains the major adult α1- and β1-globin genes (3) also hybridized

to tadpole globin cDNA. When the restriction maps of λXGαβ103 and λXGαT105

were compared, it was clear that the two clones contain a common region

(Figure 4). Thus, a tadpole α-globin gene (αT1) which hybridizes strongly

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Eco RI
GAATTCCAGATTGCTCAAACAAAATCTCTTTCTGTGCAG TTG CTG TCT CAC ACC ATC
                                        leu leu ser his thr ile   X.laevis tadpole αT1
                                        leu leu ala his asn ile   X.laevis adult α1


CAG GTG ACT CTG GCC ATC CAC TTC CAT AAG GAA TTT GAT GCT GCC ACC
gln val thr leu ala ile his phe his lys glu phe asp ala ala thr   X.laevis tadpole αT1
leu val val val ala met asn phe pro lys gln phe asp pro ala thr   X.laevis adult α1


CAT GCT GCT TGG GAC AAA TTC CTG GCT GAG GTT GCC ACC GTC CTC ACC
his ala ala trp asp lys phe leu ala glu val ala thr val leu thr   X.laevis tadpole αT1
his lys ala leu asp lys phe leu ala thr val ser thr val leu thr   X.laevis adult α1


TCC AAG TAC AGA TAA AGAAAGAAGACATCAGCAAGGGCAGCTCACCAGCTTGGGGAAGATCT
ser lys tyr arg                                             Bgl II
ser lys tyr arg
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Fig. 3.   The DNA sequence of exon 3 from a tadpole α globin gene (αT1).
            The sequence is presented for the Eco R1-Bgl II fragment at the
            3'-end of αT1 (Fig, 4). The sequence was determined in both
            strands from the Eco R1 and Bgl II sites. The amino acid sequence
            has been derived from the DNA sequence for comparison with the
            X. laevis adult α1 globin sequence (R.M. Kay, R. Harris, R.K.
            Patient and J.G. Williams, unpublished data). Residues in common
            with the human α globin sequence (1) are underlined. Nucleotides
            conserved at intron/exon boundaries are also underlined (26).
            When we compare the restriction maps and nucleotide sequence of
            our αT1 and the larval α1 of Widmer et al., (25), we find a
            number of differences. In particular, two nucleotide differences
            in the third exon give rise to two different amino acids which,
            in αT1, are conserved in human or X. laevis adult α globins.
            These differences give rise to concern but may reflect genuine
            polymorphism. Certainly restriction site polymorphisms outside
            the structural genes are quite common in X. laevis (2, 3, Fig. 4),
            but the differences mentioned here would represent the first
            coding sequence polymorphisms reported for X. laevis. An alter-
            native explanation could be that the two genes are not homologues.

to tadpole globin cDNA is linked to the major adult α-globin gene (α1) and
separated from it by 5.2 kb.

     The restriction map of another genomic clone (cXGαβ4.2) containing an
additional ∿6 kb of DNA downstream of β1 is depicted in Figure 4. This clone
was isolated from a library produced by partial digestion of X. laevis DNA
with Eco R1 and ligation into the cosmid vector Homer I (7). The insert in
cXGαβ4.2 was unexpectedly small (22.7 kb) because of the presence of three
vector molecules arranged in a head-to-tail fashion. No globin genes were
found within ∿8 kb downstream of β1 (Figure 4).

3.   The αT2 tadpole globin gene is linked to α2

     The clone λXGα102 which contains the minor adult α-globin gene, α2, also
hybridizes to tadpole globin cDNA. In order to determine whether the tadpole
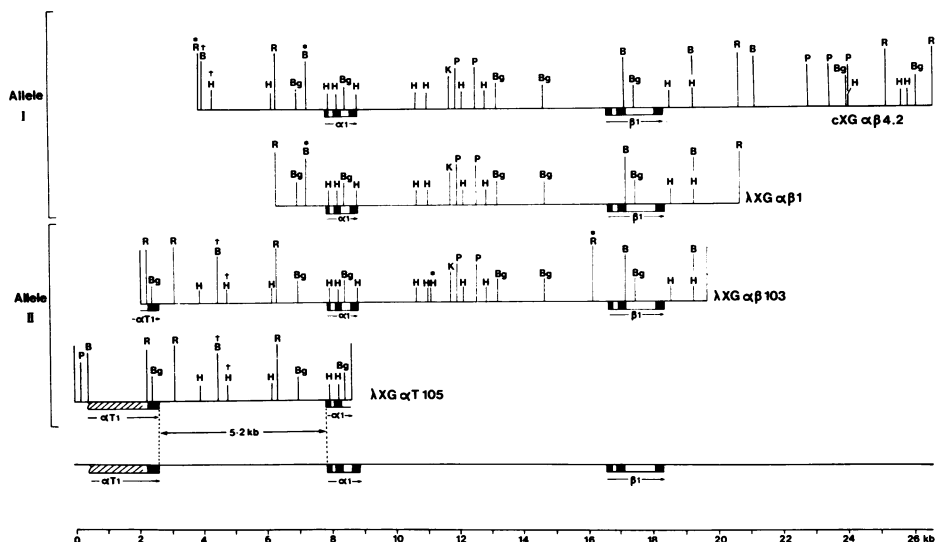
Fig. 4.   Restriction maps of two alleles of the major globin locus in
           X. laevis. Symbols are defined in the legend to Fig. 2. The
           structure of the αT1 gene was determined by DNA sequencing and
           Southern blotting with tadpole globin cDNA. Restriction sites
           not present in both alleles are marked with an asterisk or, for
           some of the sites between αT1 and α1, with a dagger. The latter
           region is distinguished in this way because, since the Bam HI and
           Hind III sites retain the same relationship to each other in both
           alleles, a deletion/insertion event may be implicated.

globin gene present in this clone is an α- or β-globin, the small Eco R1

fragment from λXGαT105, which contains the 3' exon of αT1, was purified,

labelled by nick-translation and hybridized to λXGα102 DNA spotted onto a

nitrocellulose filter (Figure 5). The level of hybridization with λXGα102

was very similar to that with λXGαβ103 and λXGαT105, both of which contain

the fragment used as probe. This indicates that the tadpole globin gene

in λXGα102 is an α globin gene. This conclusion was further reinforced when

a filter containing the same clones as in Figure 5 was hybridized with a

tadpole β globin cDNA clone. λXGα102 failed to hybridize but the clone

λXGβT106, which we know to contain a tadpole β globin gene, hybridized very

strongly (D. Banville, unpublished observations). Southern blot analysis of

λXGα102 showed that the restriction fragment which hybridized with tadpole

cDNA, also hybridized with the small Eco R1 fragment used in the above

dot blot experiment (data not shown). The restriction map derived from this

analysis shows that the tadpole α globin gene in λXGα102 (αT2) lies 10.7 kb
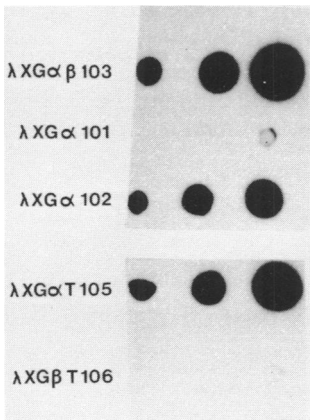
Fig. 5. DNA dot blots with the small Eco R1 fragment from λXGαT105, containing exon 3 from αT1. Each phage DNA was loaded at three levels differing by a factor of 10.

λXGαβ 103

λXGα 101

λXGα 102

λXGαT 105

λXGβ T106

upstream of the adult α2 globin gene (Figure 2).

DISCUSSION

1.   Genes encoding minor adult globin proteins

The minor globin locus of X. laevis, which contains the α2 and β2 genes, was detected by Southern blotting using the α1 and β1 cDNA clones as probes and was assumed to encode minor globin polypeptides (2). Our HART analysis with λXGα102 strongly suggests that the α2 globin gene is indeed expressed. Similarly, the cloning of pXG282 and its correlation with a polypeptide by HART (6) and with the β2 gene by Southern blot analysis (A. Jeffreys, per-sonal communication) suggest that the β2 globin gene is also expressed. The HART analysis with λXGα101 identifies an α3 gene product and thus, at least five of the six detectable polypeptides are coded for by separate genes. The chromosomal location of α3 and β3 is currently under investigation.

2.   Genes encoding tadpole α globin proteins

We have shown that a tadpole α globin gene (αT1) is linked to the major adult globin locus (α1-β1). Similarly, we have shown that another tadpole α globin gene (αT2) is linked to the minor adult globin locus (α2-β2). The similar arrangement at these two loci reinforces our previous suggestion that they arose by genome duplication (2). However, the separation between αT1 and α1 is 5.2 kb whereas the distance between αT2 and α2 is 10.7 kb. A possible explanation for this difference is the occurrence of an insertion/deletion between the αT and α genes at some time since the gene duplication which gave rise to them. There is however another possible explanation.

The Tm values of the heteroduplexes formed between the α1 and β1 cDNA

clones and the $\alpha2$ and $\beta2$ genes differ from the Tm values of the correspond-
ing homoduplexes by $\sim8^{o}$ (2). This corresponds to $\sim8\%$ nucleotide sequence
divergence (27). A similar value was obtained by Widmer et al., (25) for
four cDNA clones of $\alpha1$, $\beta1$, $\alpha2$ and $\beta2$. Similar extents of divergence have
been found for both the A and B vitellogenin cDNA clones (5%, 28) and for
two albumin cDNA clones (8%, 29). This suggests that all these gene pairs
arose as a result of tetraploidization. Widmer et al., (25) have also
isolated cDNA clones derived from four different tadpole globin mRNA
sequences; two encoding α globin polypeptides and two encoding β-globin
polypeptides. The nucleotide sequence divergence of each pair (13-14%) is
significantly greater than the adult gene pairs discussed above. This
suggests that either the larval genes have diverged more rapidly since the
genome duplication or they underwent a gene duplication prior to the genome
duplication with subsequent loss of a larval α and β globin gene at each
locus (30). If the latter model is correct then we can present a testable
hypothesis for α globin gene evolution in X. laevis.

1. An initial αT-α pair underwent a gene duplication to give rise to
    αT-αT-α and gene divergence yielded the structure αT2-αT1-α1.

2. Genomic duplication occurred during tetraploidization.

3. At some stage after this, a different one of the two αT genes at each
    locus was inactivated. This predicts the structures $\psi\alpha$T2-αT1-α1 and
    αT2-$\psi\alpha$T1-α2. Such a model is testable by careful searching of the
    sequences between αT2 and α2 for vestiges of an αT1 globin gene and
    such an analysis is under way.

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