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A *Xenopus* homologue of *aml-1* reveals unexpected patterning mechanisms leading to the formation of embryonic blood

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SUMMARY

The Runt domain gene *AML1* is essential for definitive hematopoiesis during murine embryogenesis. We have isolated *Xaml*, a *Xenopus AML1* homologue in order to investigate the patterning mechanisms responsible for the generation of hematopoietic precursors. *Xaml* is expressed early in the developing ventral blood island in a pattern that anticipates that of later *globin*. Analysis of *globin* and *Xaml* expression in explants, in embryos with perturbed dorsal ventral patterning, and by lineage tracing indicates that the formation of the ventral blood island is more complex than previously thought and involves contributions from both dorsal and ventral tissues. A truncated Xaml protein interferes with primitive hematopoiesis. Based on these results, we propose that Runt domain proteins function in the specification of hematopoietic stem cells in vertebrate embryos.

Key words: Hematopoiesis, Stem cell, Runt domain, *Xenopus*, Fate map, Gastrula, Blood island, *Xenopus acute myeloid leukemia* (*Xaml*).

INTRODUCTION

An understanding of the molecular events leading to the formation of hematopoietic stem cells (HSC) in the early embryo will have profound medical and therapeutic applications. HSCs are formed as a downstream consequence of the earliest patterning events of embryonic development that lead to the formation of the anterior/posterior, dorsal/ventral and left/right axes. As much of the understanding of vertebrate axial patterning comes from the *Xenopus laevis* model system, we chose to study hematopoiesis in *Xenopus.* In a normal *Xenopus* embryo, primitive erythrocytes form in a highly reproducible and stereotypical fashion in a ventral location of the tailbud embryo called the ventral blood island (VBI). The VBI initially forms in a somewhat anterior position on the ventral surface of the embryo and assumes a V-shaped morphology. As hematopoiesis continues, additional primitive erythrocytes are formed in a wave-like progression as the VBI spreads posteriorly from its initial V-shaped morphology (Kelley et al., 1994). The embryonic patterning events that result in this highly localized expression of the blood program are unknown. However, it is widely believed that primitive erythrocytes in *Xenopus* are derived strictly from the ventral marginal zone (VMZ) of the early gastrula. Several pieces of experimental evidence support this idea (Hemmati-Brivanlou and Thomsen, 1995; Maeno et al., 1985, 1992; Mead et al., 1996).

Recent evidence indicates that the transcription factor CBF plays an important role in embryonic hematopoiesis. CBF is a heteromeric factor whose DNA-binding α subunit is encoded by three closely related genes *cbfa1*, *cbfa2* and *cbfa3.* An unrelated partner protein termed CBFβ enhances the DNA-binding affinity of the α subunit. The CBF α subunit genes are known as Runt domain genes (RDG; Speck and Stacey, 1995). Members of this gene family contain a highly conserved 128 amino acid motif named the 'Runt domain' after the *Drosophila* gene *runt*, which was the first RDG to be cloned and characterized (Daga et al., 1992; Kagoshima et al., 1993). The Runt domain is required for interaction with the β subunit as well as for the interaction with DNA (Golling et al., 1996; Kagoshima et al., 1993) . Initial indications that CBF functions in hematopoiesis came with the discovery that genes encoding both subunits of CBF are mutated in human leukemias. The human homolog of *cbfa2* is one of the most commonly affected genes in translocations associated with acute myeloid leukemia and is therefore termed *aml1* (Miyoshi et al., 1991). More recently, *cbfb* has also been found to be mutated in leukemias (Liu et al., 1993). Targetted mutations in transgenic mice demonstrate that the *cbfa2* and the *cbfb g*enes are required for the development of all definitive blood lineages but are not essential for the formation of the primitive lineages (Wang et al., 1996a,b). This phenotype suggests that these genes are required very early in the definitive lineage but they are not required for the specification of the precursor of the primitive lineages.

Here, we extend the study of RDGs to *Xenopus.* We have isolated a *Xenopus* homolog of *cbfa2/aml1* and have named this gene *Xaml* for *Xenopus acute myeloid leukemia*. Our analysis of *Xaml* expression and function indicates that the ventral blood island in *Xenopus* has a complex embryonic

origin and requires inputs from both dorsal and ventral tissues. In addition, our results indicate a requirement for runt domain genes in primitive hematopoiesis. CBF thus appears to be required for the development of all blood lineages and may function in the early formation of the HSC.

MATERIALS AND METHODS

Library screening and cloning procedures

A λgt11 *Xenopus* stage 17 cDNA library was plated and screened using standard laboratory procedures (Sambrook et al., 1989). To prepare the *cbfa2* runt domain, DNA probe pSPS4TS (Bae et al., 1994) was digested with *Sma*I and *Bgl*II, and the 600 bp insert containing the encoded runt domain was purified. This DNA was then radiolabeled using a Random Primer DNA Labeling Kit™ (Boehringer-Mannheim). Hybridizations were performed overnight and the filters were washed at low stringency (2× SSC, 0.1%SDS at 50°C). Three positive plaques were isolated and purified to homogeneity. The DNA was purified from these clones and the inserts were subcloned into pBSK+ (Stratagene) for sequencing. Only one of these subclones (p5.31) was found to encode a runt domain gene.

The XRD construct was created by PCR amplification of a portion of p5.31 using the upstream primer 5′-GGAATTCCACCATGGTGGAAGTATTG-3′ and the downstream primer 5′-GCTCTAGAGCTCATCGCCGTGGCTCGCGAGG-3′. Following amplification the Runt domain DNA was subcloned into an *Eco*RI/*Xba*I-digested pCS2+ expression vector to generate pXRD-8. To generate capped mRNA, pXRD-8 was linearized with *Pvu*II and transcribed in vitro using the SP6 mMessage mMachine ™ (Ambion). For functional analyses, full-length *Xaml* was subcloned into pCS+2 to generate pCSX. The *Xaml* mRNA for injecting embryos was prepared by linearizing pCSX with *Not*I and in vitro transcription with SP6 polymerase.

Whole-mount in situ hybridizations and lineage tracing

The in situ hybridizations were performed using digoxigenin-labeled RNA probes and the BM-Purple substrate (Boehringer-Mannheim). The antisense *Xaml* probe was generated by cleaving p5.31 with *Sal*I followed by in vitro transcription with T7 polymerase. The antisense *αT1 globin* probe was generated as described previously.

For lineage tracing experiments, *lacZ*-injected embryos were fixed in 1× MEMFA (0.1 M MOPS (pH 7.4), 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde) for 1 hour after manual removal of the vitelline envelope. Embryos were then washed twice for 5 minutes in 0.1 M sodium phosphate buffer (pH 6.3). The color reaction was performed in 0.1 M phosphate buffer + 0.01 M potassium ferricyanide + 0.01 M potassium ferricyanide + 0.15% X-gal. After the blue color was sufficiently developed, embryos were stored in 100% ethanol until needed for whole-mount in situ hybridization.

RT-PCR

RT-PCR was performed using a primer (5′-CTGGGGAGAGTACATAGGTG-3′) that anneals with the 5′ untranslated region of *Xaml* and a downstream primer (5′-AGTGGTGGAGGGCGGCGTGAAG-3′) that anneals within the *Xaml* coding region. This primer combination yields a 243 bp product. All reactions were carried out with an annealing temperature of 60°C and for 32 cycles.

Ultra-violet irradiation (UV) and lithium treatment

UV treatment of the vegetal pole was at 28 minutes postfertilization and stopped after 120 seconds. All UV treatment experiments used a 245 nm UV light source. This treatment produces embryos varying from DAI=2 to DAI=0. LiCl treatment was in 0.1× Mark's Modified Ringers (MMR) (1× MMR = 100 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 1 mM MgCl2, 5 mM Hepes, pH 7.4) containing 0.3 M LiCl at the 32- to 128-cell stage. To ensure variable levels of dorsalization, we removed embryos from the LiCl solution at 2.5 minute intervals. Embryos were exposed to LiCl for 5, 7.5, 10 or 12.5 minutes.

Embryonic explants

All explants were performed on stage 10 embryos with both microsurgery and healing performed in 0.5× MMR. Ventral explants consisted of a 30° arc opposite to the dorsal blastopore lip. Dorsal explants consisted of a 30° arc centered around the dorsal blastopore lip and included presumptive ectoderm of the animal cap to promote healing; the majority of the attached endoderm was removed but the endomesoderm of Brachets cleft was left intact. In dorsal explants that had the endomesoderm removed, this tissue was carefully dissected away from the presumptive involuting mesoderm.

Microinjections

In experiments with pigmented embryos, we injected either the two dorsal or two ventral blastomeres of 4-cell embryos with 5 nl of a 400 pg/nl solution containing either *XRD* or *Xaml*, and either *lacZ* or empty vector (CS2+) mRNA. For scoring of the circulating blood, embryos were anaesthetized with benzocaine and observed under a dissecting microscope at high magnification (see text). When lineage tracing, we injected both blastomeres of 2- to 4-cell embryos with a solution containing either 50 pg/nl *lacZ* + 150 pg/nl *XRD*, or 50 pg/nl *lacZ* alone.

Northern blotting and benzidine staining

In northern blotting experiments, total RNA was extracted from bloodless and control embryos by incubation with proteinase K followed by DNAase treatment and two phenol chloroform extractions and ethanol precipitation. The antisense α*T1globin* and *histoneH4* probes were prepared as described previously. Benzidine staining was performed on stage 50 larvae as described previously (Hemmati-Brivanlou and Thomsen, 1995).

RESULTS

Cloning of *Xenopus acute myeloblastic leukemia-1*

To identify homologues of *aml-1* expressed early in embryonic development, we screened a *Xenopus* neurula (stage 17) cDNA library at low stringency with a probe consisting of the runt domain of mouse *cbfa2*. We identified one clone (clone 5.31) encoding a protein highly similar to human *aml-1* and mouse *cbfa2* (Fig. 1). This gene was designated *Xaml* for *Xenopus acute myeloid leukemia*. The 2027 base pair insert of clone 5.31 encodes a single long open reading frame (ORF) of 461 amino acids representing a full-length XAML protein. By sequence alignment, the XAML protein is most similar to that produced by the most abundant transcript from the murine *cbfa2* gene. The proteins are 90% identical overall, with only three conservative amino acid substitutions in the runt domain. In contrast, the murine *cbfa1* and *cbfa3* runt domains differ from that of XAML at twelve positions.

Embryonic expression of *Xaml*

Using the reverse transcriptase polymerase chain reaction (RT-PCR) to detect the *Xaml* mRNA, we detect a small maternal component of *Xaml* in 2-cell embryos (Fig. 2A). *Xaml* transcription begins at stage 8, which is when zygotic transcription initiates in *Xenopus* and increases steadily after this time point (Fig. 2A). We first detect *Xaml* expression using whole-mount in situ hybridization at Nieuwkoop-Faber stage 14.

Whole-mount in situ hybridization reveals that *Xaml* mRNA is expressed in a complex and dynamic pattern. In stage 14-24 embryos, *Xaml* is expressed in a subset of neuroblasts that are found in the lateral stripe of the neural plate (Fig. 2B). In late neurula stages, *Xaml* expression begins to be expressed in the olfactory placodes (Fig. 2G). The first *Xaml* expression that appears likely to be involved in blood formation is seen on the anterior ventral side of the embryo at stage 14 (Fig. 2D). Closer inspection of this expression domain in sectioned material shows *Xaml*-positive cells are in the outer layer of anterior endomesoderm (Fig. 2C). In sections, this staining is primarily nuclear suggesting the *Xaml* mRNA turns over very rapidly and can only be detected in actively transcribing cells. This domain of *Xaml* expression is highly dynamic. As the *Xenopus* embryo elongates, the pattern of *Xaml* expression shifts gradually from a patch of cells in the anterior endomesoderm underlying the cement gland anlage at stage 14 to a V-shaped expression pattern in the presumptive VBI as early as stage 22 (Fig. 2D-F). This changing pattern of expression could be due to changes in gene expression or to an anterior-posterior migration of *Xaml*-expressing cells. At later stages, when embryonic *α-globin* expression can first be detected, it is in a V-shaped pattern (Fig. 2H) like *Xaml* (Fig. 2G). However, *Xaml* at these stages differs from *α-globin* in that expression is seen in cells anterior to the VBI, in lateral plate mesoderm (Fig. 2I), as well as posterior to the domain of *α-globin*-expressing cells in similarly staged embryos (Fig. 2G,H). This more posterior *Xaml* expression is predictive of future *α-globin* expression at later stages and continues to expand as the embryos age until it reaches its' posterior boundary at the proctodeum at approximately stage 26.

The expression of *Xaml* in the VBI in a pattern that anticipates the pattern of *α-globin* expression suggests that *Xaml* plays a role in primitive hematopoiesis. A simple model to explain the dynamic pattern of *Xaml* expression described above is that the VBI comes to be populated by *Xaml*-expressing cells, which originate in the anterior endomesoderm. However, this model poses a dilemma. Anterior endomesoderm is dorsally derived (Fig. 3) (Keller, 1991; Vodicka and Gerhart, 1995), whereas primitive blood is thought to develop solely from ventrally derived cells. Therefore, to investigate the role of *Xaml* in hematopoiesis, we felt it was essential to clarify the origin of *Xaml*-expressing cells and their relationship to hematopoietic precursors. As we will show below, *Xaml*-expressing cells in the VBI have two separable origins. Early expression in the anterior of the VBI is in dorsally derived cells, whereas expression in the posterior VBI at later stages is in ventrally derived cells. We will refer to these separable domains of *Xaml* expression as EA (early anterior) (Fig. 3) and LP (late posterior) for the remainder of the paper.

Expression of *Xaml* in tissue explants

As a first test to determine the origin of *Xaml*-expressing cells, we analyzed DMZ explants and VMZ explants. In this experiment, presumptive dorsal or ventral tissues are dissected from the embryo at the earliest stages of gastrulation and then cultured in vitro until later developmental stages (Fig. 3). *Xaml* expression is present in control stage 17 DMZ explants but not in VMZ explants (Fig. 4A,B). Importantly, the pattern of *Xaml* expression in DMZ explants is similar to the EA *Xaml* pattern observed in intact embryos. Since our analysis of whole embryos showed EA *Xaml* was localized to anterior endomesoderm, we included endomesoderm in these explants. When we analyzed DMZ explants with the endomesoderm removed, we did not detect *Xaml* expression (data not shown). Based on these results, the dorsal endomesoderm and not the ventral marginal zone accounts for the EA domain of *Xaml* expression.

At later stages, *Xaml* expression in the VBI spreads posteriorly from the early V-shaped pattern of expression. This changing pattern of expression could be due to migration of dorsally derived cells or to de novo expression of *Xaml* in ventrally derived cells. To distinguish between these possibilities, we examined *Xaml* expression in DMZ and VMZ explants at stage 25-27. At these later stages, *Xaml* expression is seen in VMZ explants but not in DMZ explants (Fig. 4C,D).

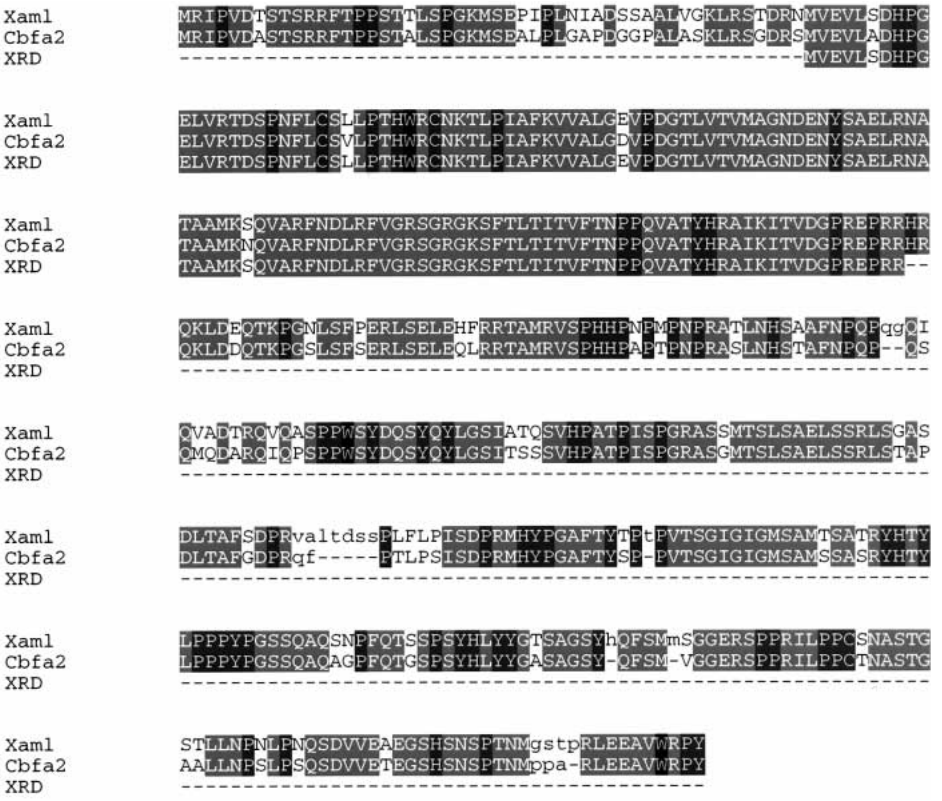

Fig. 1. XAML, CBFa2 and XRD sequence alignment. The predicted amino acid sequence for XAML (GenBank accesion number AF035446) is approximately 90% identical to CBFa2. Insertion/deletions and regions of the proteins which cannot be aligned are lower case. The darker shading indicates a high pair-mean score (obtained by the Macaw sequence alignment program).

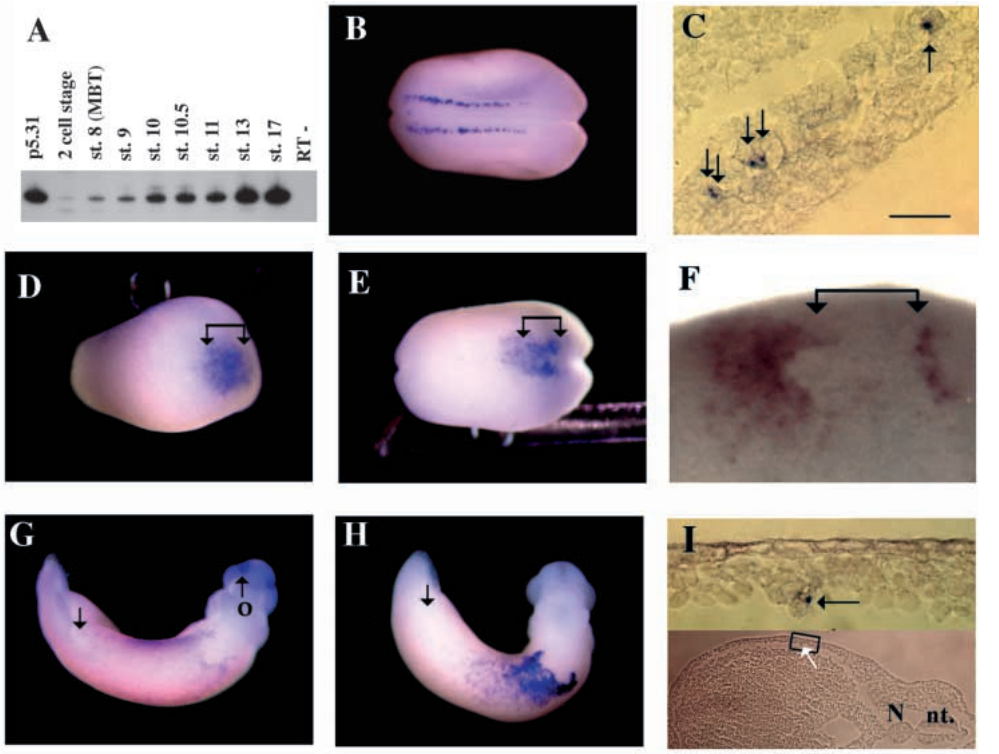

Fig. 2. Expression of *Xaml* in *Xenopus* embryogenesis. *Xaml* (A-G,I) and *α-globin* (H) expression patterns, in all panels anterior is to the right. Bracket in D-F, 400 μm. Arrows indicate *Xaml*-expressing cells (C,I). (A) RT-PCR of Xaml expression at different embryonic stages. (B) Dorsal view of *Xaml* expression in neuroblasts at stage 17. (C) Sagittal section through anterior endomesoderm of a stage 14 embryo, *Xaml* is in cells of the endomesoderm which make direct contact with the ectodermal layer, Bar, 20 μm. (D) Ventral view of a stage 14 embryo. (E) Ventral view of embryo shown in B; note that *Xaml* expression has expanded posteriorly. (F) Detail of EA *Xaml* expression at stage 22. At this stage *Xaml* expression refines into a V-shape, but some *Xaml* cells remain anterior to the VBI (right arrow). (G) Ventral *Xaml* expression in a stage 26 embryo extends to the proctodeum (arrow). (H) *α-globin* expression in a stage 26 embryo, has not yet reached the proctodeum (arrow) demonstrating that *Xaml* expression precedes *α-globin* in the VBI. (I) Transverse section of a stage 26 embryo at approximately 30% embryonic length. *Xaml* expression is seen in isolated cells of the lateral plate mesoderm, top panel is a high magnification view of the region boxed in the lower panel. Abbreviations: N, notochord, nt, neural tube, o, olfactory placode.

These explants were processed exactly as those described above (Fig. 4A,B) except that they were allowed to develop in vitro for a longer period of time. This result indicates the posterior expansion of *Xaml* expression is due, at least in part, to de novo expression in ventrally derived cells. It is this ventral aspect of *Xaml* expression that we refer to as LP *Xaml*. *Xaml* expression in both dorsal and ventral explants contrasts with *α-globin* which is expressed only in ventral explants (Hemmati-Brivanlou and Thomsen, 1995; Maeno et al., 1985, 1992, 1996).

Sensitivity of *Xaml* and *α-globin* to perturbations of dorsal/ventral patterning

While tissue explant experiments examine the potency and commitment of the tissue of interest, they do not address interactions of tissues that normally occur in an intact embryo. In *Xenopus* it is possible to study the effects of dorsal/ventral patterning in the context of the whole embryo by experimental treatment with ultraviolet radiation (UV), which ventralizes embryos (Elinson and Pasceri, 1989), or with LiCl, which dorsalizes embryos (Kao and Elinson, 1988; Klein and Melton, 1996). By altering the level of UV treatment or LiCl treatment, a phenotypic series of transformations can be generated which is scored according to a dorsoanterior index (DAI). On this scale, normal embryos have a DAI=5, completely ventralized embryos have a DAI=0 and completely dorsalized embryos have a DAI=10 (Kao and Elinson, 1988).

If EA *Xaml* expression occurs in cells that are dorsally derived then *Xaml* expression should be increased by LiCl treatment. As expected, the number of *Xaml*-expressing cells is greatly increased in DAI 8 dorsalized embryo (Fig. 5A,D). Interestingly the *Xaml*-expressing cells in DAI 8 embryos extends from the heart primordium into a region of the embryo that would be the VBI in a normal embryo. However, *Xaml* expression is eliminated by extreme dorsalization and begins to decrease as the DAI exceeds 8 (Fig. 5A). The response of *Xaml* to LiCl is therefore dependent on the level of dorsalization. At low LiCl doses *Xaml* expression is increased but at high doses *Xaml* expression is decreased.

The dorsal nature of the EA domain of *Xaml* expression is confirmed in UV ventralized embryos. Early *Xaml* expression in the VBI is completely eliminated by UV even in embryos that are only partially ventralized (DAI=2; Fig. 5B). In these

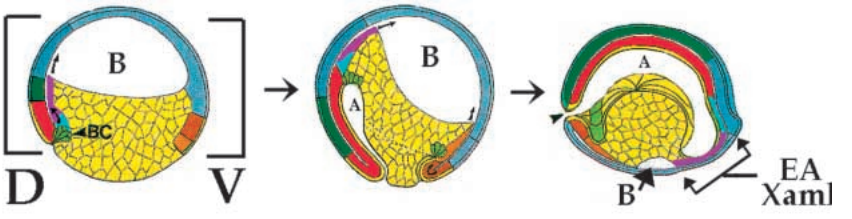

Fig. 3. Schematic representation of gastrulation in *Xenopus*. Presumptive EA *Xaml*-expressing cells are colored magenta. The embryo shown on the left is a stage 10 embryo; the brackets indicate the region of the embryo excised in explant experiments. The middle embryo shows a mid-gastrulation stage embryo. The embryo on the right is at a similar stage to the earliest that we detect *Xaml* by whole-mount in situ hybridization. Abbreviations: A, archenteron, B, blastocoel cavity; BC, bottle cells; D, dorsal; V, ventral. (Adapted from Keller, 1991).

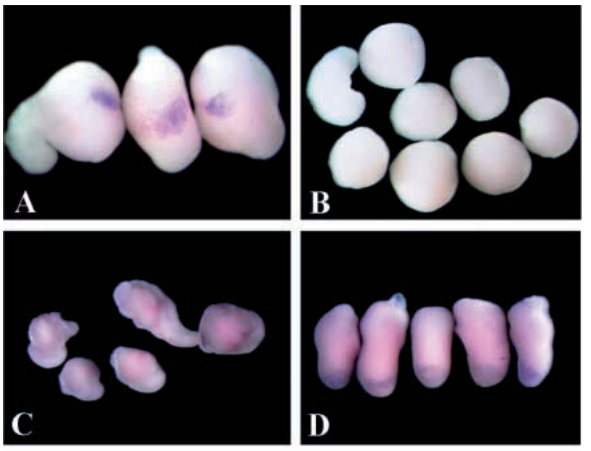

Fig. 4. *Xaml* in situ hybridization in dorsal and ventral explants. (A) Dorsal explant at control stage 15-17 showing positive *Xaml* staining. The position of *Xaml* expression is in a ventral location relative to the head folds, similar to the position of EA *Xaml* in a whole embryo. (B) Ventral explant at control stage 15-17 with lack of *Xaml* staining. (C) Dorsal explant at control stage 25-27 with lack of *Xaml* staining. (D) Ventral explant at control stage 25-27; note intense *Xaml* staining at the 'anterior' tip (pointing down) of the explant, this location of staining is similar to that seen with an *α-globin* probe (data not shown).

partially ventralized embryos, *Xaml* expression is still seen in a subset of neuroblasts (Fig. 5C) which serves as a positive control for the in situ hybridization. More importantly, this result indicates that EA *Xaml* expression in the VBI is extremely sensitive to UV, which is contrary to expectations if EA *Xaml* expression is ventrally derived.

The differential sensitivity of EA *Xaml* expression to UV treatment and LiCl treatment is consistent with the results of our explant experiments. Together these experiments demonstrate that EA *Xaml*-expressing cells in the VBI are dorsally derived. As blood is traditionally viewed as a ventrally derived tissue in the early embryo, it thus appeared as if EA *Xaml* expression might be irrelevant to primitive hematopoiesis. However the pattern of *Xaml* expression, especially when compared to*α-globin* suggested this question required further investigation.

Surprisingly, we found that treatment of embryos with LiCl affected *α-globin* expression in a manner that exactly paralleled that of *Xaml* expression. Highly dorsalized embryos had greatly reduced *α-globin* expression while embryos with intermediate dorsalization showed high levels of *α-globin* expression (Fig. 5E). Intense *α-globin* expression is seen in the ventral-most region of the DAI 8 embryo. The *α-globin* expression domain overlaps with the *Xaml* expression domain but there are a large number of *Xaml*-expressing cells anterior

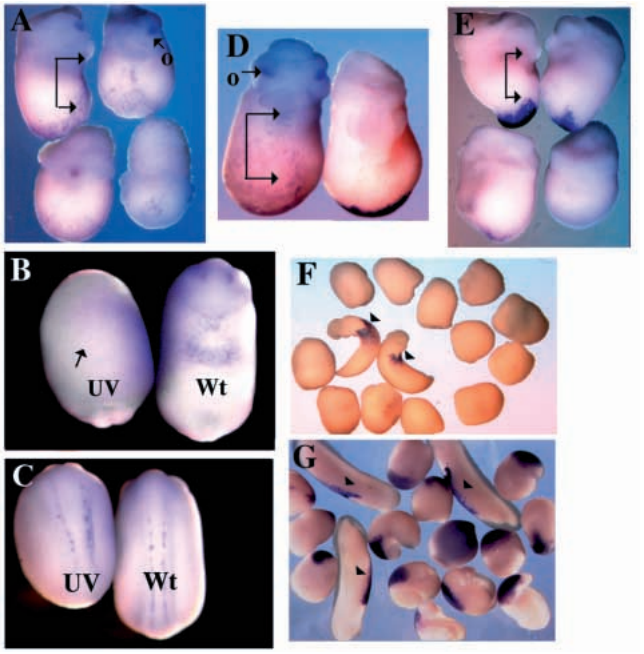

Fig. 5. Comparison of the effects of UV ventralization and LiCl on *Xaml* (A-D) expression and *α-globin* (D-G) expression. Arrowheads indicate untreated controls in F and G. The bracket indicates the region of a DAI=8 embryo where *Xaml* is increased but *α-globin* is absent (A,D,E). (A) LiCl-treated embryos at control stage 27 following in situ for *Xaml*. The top two embryos are DAI=8 the bottom two embryos are DAI=9. (B)Ventral view of an embryo probed for *Xaml* expression after UV treatment (left embryo); the arrow indicates the region of the UV embryo where *Xaml* expression is missing. A wild-type control embryo is shown on the right. (C) Dorsal view of embryos shown in B (see text). (D) LiCl embryos at control stage 27 (DAI=8) following whole-mount in situ hybridization for *Xaml* (right embryo) or *α-globin* (left embryo). (E) LiCl-treated embryos at control stage 27 following *αT1 globin* in situ. The top two embryos have DAI=8; the bottom two embryos have DAI 9. (F) *α-globin* expression in UV ventralized embryos at control stage 26 (G) *α-globin* expression in UV ventralized embryos at control stage 28-29. Abbreviations: o, olfactory placode; WT, untreated control.

to the region that expresses *α-globin* (Fig. 5A,D,E). This situation is reminiscent of what is seen in untreated embryos where a few *Xaml*-expressing cells can normally be seen anterior to the domain of *α-globin* expression.

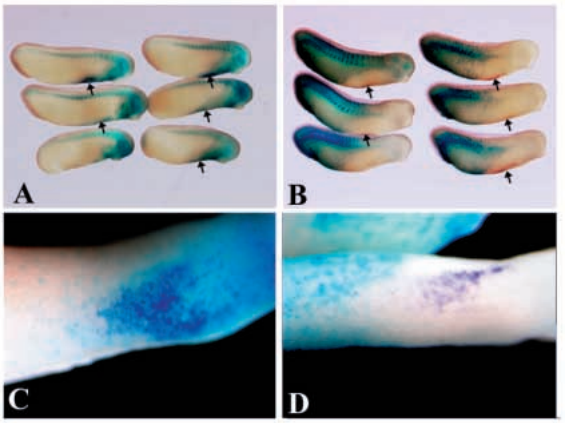

Fig. 6. *α-globin* expression in embryos lineage traced with *lacZ* following in situ hybridization with an *αT1 globin* probe. β-gal staining gives a blue precipitate while *α-globin* staining is purple. (A) Embryos with a β-gal staining pattern typical of dorsally injected pigmented controls. Arrowhead indicates *α-globin* expression. (B) Embryos with β-gal staining typically seen in ventrally injected pigmented controls. Arrowhead indicates *α-globin* expression. (C) Ventral view of an embryo treated as in A. Note the significant overlap of the β-gal staining and the *α-globin* staining. (D) Ventral view of embryo shown in B.

When we analyzed the effects of UV on *α-globin* expression, we found a subtle effect that has not been noted in previous studies. As expected from previous work, globin expression was present in UV ventralized embryos. However, we found that expression of *α-globin* in these embryos occurred consistently later than in their untreated sibs. Expression of *α-globin* in UV-treated embryos is not detected at stages when their untreated sibs express *α-globin* in the anterior VBI (Fig. 5F). Our interpretation of this result is that early *α-globin* expression is eliminated by UV because it has a dorsal origin, whereas the ventrally derived *α-globin* expression observed at later stages is unaffected by UV (Fig. 5G). Interestingly, we found that *Xaml* expression at later stages is also unaffected by UV and is in a similar pattern to *α-globin* (data not shown). This is consistent with the results of explants experiments demonstrating that EA *Xaml* is in dorsally derived cells and that LP *Xaml* is in ventrally derived cells. These observations also further strengthen the correlation in the expression patterns of *Xaml* and *α-globin*.

Early *α-globin* expression overlaps dorsally derived cells

Although the results of our explant experiments and our UV versus LiCl experiments indicate EA *Xaml* cells have a dorsal origin, there are inherent limitations to both of these approaches. Therefore to further confirm the origin of anterior *Xaml*-expressing cells, we performed lineage tracing experiments by injecting *lacZ* mRNA into the cleavage furrow near the marginal zone of 2-cell-stage albino embryos. Approximately half of the embryos injected in this fashion will be labeled by *lacZ* in dorsoanterior endo/mesoderm and half of the embryos will be labeled in ventral mesoderm. As albino embryos are not pigmented, it is not possible to know at the time of injection whether or not a particular embryo has been injected dorsally or ventrally. Therefore, as a control to determine the pattern of labeling for dorsal versus ventral injections, we also injected pigmented embryos in either the dorsal marginal zone or the ventral marginal zone, respectively. In these experiments, we found that the *lacZ* injected in the DMZ labeled anterior ventral mesoderm while only the posterior ventral mesoderm was labeled in ventrally injected embryos.

In our experiments, injected albino embryos were allowed to develop to early neurula stages and stained for β-galactosidase (β-gal) activity using X-gal followed by in situ hybridization with an antisense *Xaml* probe. We found that β-gal staining overlapped EA *Xaml* staining only in embryos that had staining indicative of *lacZ* injection in the dorsal marginal zone (data not shown). Interestingly, dorsal injections with *lacZ* appear to label a population of cells in the presumptive anterior ventral blood island, which prompted us to perform the same experiment at later stages using an *α-globin* probe. Strikingly we found that early *α-globin* expression overlaps with β-gal staining indicative of dorsal injections (Fig. 6A,C). In contrast, we found that early *α-globin* expression in stage 25 embryos does not overlap β-gal staining in ventrally injected embryos (Fig. 6B,D).

This lineage tracing technique does not allow for single cell level of resolution. Formally, the conclusion from this experiment is that early *α-globin* expression overlaps and is in very close proximity to dorsally derived cells. We cannot conclude that the *α-globin*-expressing cells themselves are dorsally derived. However this lineage tracing experiment indicates that the early *α-globin*-expressing cells do not originate in the VMZ as we never observed strong lacZ staining overlapping with *α-globin* in ventrally injected embryos. The virtually complementary staining patterns observed with dorsal versus ventral injections argue strongly that the early *α-globin*-expressing cells in intact embryos are dorsally derived.

A truncated XAML protein inhibits primitive hematopoiesis

Given the striking correlation between *Xaml* expression and primitive *α-globin* expression in the experimental manipulations described above, we wished to examine the possibility that *Xaml* functions in primitive hematopoiesis. To do this, we wished to construct a XAML protein that would function as a dominant negative mutant. We thought a protein consisting solely of the *Xenopus* runt domain (XRD) might accomplish this goal (Fig. 1). The runt domain is necessary and sufficient for both DNA binding as well as for binding to the partner protein, CBFβ (Kagoshima et al., 1996). However, sequences outside the runt domain are necessary for both *trans*-activation and repression activities of runt family members (Aronson et al., 1997; Bae et al., 1994). Thus, XRD should compete with the endogenous XAML for CBFβ and for the *cis*-acting elements in genes that are normally regulated by XAML.

In our initial studies, we tested the effects of injecting mRNA encoding XRD into 4-cell-stage embryos in the marginal zone of either ventral blastomeres or dorsal blastomeres. Embryos were then allowed to develop to stage 38-40 where blood can easily be seen circulating through veins and arteries of the tail and gill arches. We found that embryos injected with *XRD* in either the VMZ or the DMZ consistently contained individuals that had reduced numbers of circulating blood cells when compared to controls. Although the embryos injected with *XRD* contained some circulating cells, for convenience we will refer to the phenotype resulting from *XRD* injection as the bloodless phenotype. An embryo was considered bloodless if there were only a few blood cells seen in the circulation occasionally passing through the field of view. In bloodless embryos there were clear interruptions in the flow of blood cells ranging from one to several seconds. This is in stark contrast to control embryos where blood cells form a continuous stream circulating through the field of view. Injection of Xrd mRNA in the VMZ resulted in 53% of the injected embryos displaying the bloodless phenotype (n=109), while full-length *Xaml* resulted in 5% bloodless embryos (n=100), and control mRNAs (*lacZ* or empty vector) resulted in 2% bloodless embryos (n=59). These effects were seen in five independent experiments using two different batches of mRNA. In addition to the bloodless phenotype, embryos injected with high levels of *XRD* in the DMZ develop with greatly reduced hearts and fluid-filled, bloated abdomens if allowed to develop to later stages (data not shown).

To test whether the *XRD*-injected embryos displaying the bloodless phenotype contained fewer mature red blood cells than controls, we stained the bloodless embryos with benzidine. Benzidine staining forms a blue precipitate in the presence of functional heme groups and as such is a stain for mature red blood cells. Benzidine staining of the bloodless embryos is

greatly reduced compared to that of controls (Fig. 7A-C). Examination of bloodless embryos at higher magnification showed that there are some cells present in the circulation that intensely stain with benzidine (data not shown). The weak benzidine staining seen in Fig. 7C is therefore due to a small number of cells intensely expressing hemoglobin as opposed to a large number of cells weakly expressing hemoglobin. Thus, the number of mature red blood cells is reduced in bloodless *XRD*-injected embryos. To further test whether or not our visual inspection technique accurately reflects the amount of blood present, we examined *α-globin* mRNA from these embryos. Embryos expressing the bloodless phenotype have reduced *α-globin* compared to controls (Fig. 7D).

In these experiments, the bloodless phenotype was not completely penetrant. This low penetrance was probably due to a combination of several factors including the fact that neither ventral or dorsal injections target all presumptive *Xaml*-expressing cells. To increase the resolution of our assay for blood formation, we examined the effects of *XRD* injection on blood formation at earlier stages using whole-mount in situ hybridization with an *α-globin* probe. To accurately determine which cells in the embryo received the *XRD* mRNA, we mixed a small amount of *lacZ* mRNA with the *XRD* message. Albino embryos were injected at the 2-cell stage in the cleavage furrow in the marginal zone; this results in 50% of the embryos injected in the DMZ and 50% in the VMZ. We observed a strong negative correlation between cells that receive the *XRD+lacZ* injection and *α-globin* expression. Importantly, embryos injected with *XRD+lacZ* with a β-gal pattern indicative of injection in the DMZ show primarily inhibition of anterior *αT1 globin* expression (Fig. 8F-H) while embryos injected in the VMZ show primarily inhibition of posterior *α-globin* expression (Fig. 8C-E). In *XRD*-injected embryos where β-gal activity is only seen outside of the VBI (Fig. 8B) as well as in embryos injected with *lacZ* (Fig. 8A) alone *α-globin* staining appears normal. These results strongly suggest that *Xaml* function is required for primitive hematopoiesis.

DISCUSSION

We have cloned a *Xenopus* homolog of the human gene *aml-1*, which we have named *Xaml*. Careful analysis of *Xaml* expression provides novel insights into vertebrate embryonic blood development. Strikingly, the earliest *Xaml* expression associated with VBI formation occurs in a dorsally derived population of cells. We provide several independent lines of evidence to support this model. *Xaml* expression is robust in stage 17 explants of dorsal meso/endoderm whereas it is absent in explants of ventral mesoderm taken from the same host embryo. We have also shown that V-shaped *Xaml* expression in the VBI is eliminated by UV ventralization whereas *Xaml* expression in the equivalent region of an embryo dorsalized with LiCl is expanded. Finally, using lineage tracing with *lacZ*, we demonstrated the existence of a dorsal cell population that populates the VBI. These lineage tracing experiments also provide strong evidence that early *α-globin* expression in the anterior VBI occurs in cells that are dorsally derived. β-gal staining in dorsally injected embryos overlaps with early *α-globin* expression whereas β-gal from a ventral/posterior injection does not. Although blood-island-derived hematopoietic lineages are generally thought to arise solely from the ventral and not the dorsal mesoderm, a dorsal origin for the mesoderm in the anterior region of the blood island is consistent with several other previous observations (Dale and Slack, 1987; Moody and Kline, 1990; Vodicka and Gerhart, 1995).

In further support of the view that dorsally derived cells contribute to the developing blood islands are our observations on *α-globin* expression in UV and LiCl embryos. The timing of *α-globin* expression in partially dorsalized embryos is early and correlates with the early V-shaped expression domain of *α-globin* in untreated control embryos. The timing of *α-globin* expression in UV-treated embryos occurs later and correlates with the posterior expansion of *α-globin* expression in normal embryos. The timing of *α-globin* expression in UV embryos is a parameter that was not appreciated in previous studies. The finding that *α-globin* expression is present in intermediately dorsalized embryos is in agreement with earlier work that examined GLOBIN immunohistologically (Cooke and Smith, 1988).

The tight correlation between *Xaml* expression and *α-globin* expression strongly suggests *Xaml* plays a role in primitive hematopoiesis. Our strongest evidence showing a role for *Xaml* in primitive hematopoiesis is that expression of the DNA-binding domain of *Xaml* (XRD) dominantly interferes with primitive hematopoiesis. Injection of mRNA encoding XRD results in larvae with a reduced number of circulating red blood cells when injected into either the dorsal or ventral mesoderm. By lineage tracing these injections, we have shown that XRD interferes with *α-globin* expression in a strikingly localized fashion. β-gal staining indicative of dorsal *XRD* injection shows inhibition of *α-globin* expression in the anterior VBI whereas ventral *XRD* injections inhibit posterior *α-globin*. This result definitively shows that dorsally derived tissues contribute to the development of the anterior *α-globin*-expressing cells in the VBI. However, this does not show *Xaml* is required in the *α-globin*-expressing cells themselves as it is possible *Xaml* functions in neighboring cells for the production of a secreted factor.

Although the experiments with *XRD* strongly suggest *Xaml* is necessary for primitive hematopoiesis, several observations indicate *Xaml* alone is insufficient to induce this pathway. First, *Xaml* is expressed in dorsal explants but these explants never express *α-globin.* Consistent with this, we were unable to generate ectopic *α-globin* expression by injection of full-length *Xaml* into embryos (data not shown). In addition, there are cells that express *Xaml* in the lateral plate mesoderm which do not express *α-globin.* Finally, in embryos treated with LiCl, *Xaml* expression is increased in a domain anterior to the domain of cells that express *α-globin.* If *Xaml*-positive cells are precursors of primitive red blood cells, the pattern of *α-globin* expression in this experiment suggests an additional more posteriorly derived signal is required to initiate terminal differentiation of these cells. It is possible that, in normal embryos, the interaction of dorsally derived *Xaml*-expressing cells in the anterior VBI with ventrally derived cells in the posterior VBI determines the anterior boundary of *α-globin* expression. This suggests that the anterior *α-globin*-expressing cells themselves originate dorsally or require a signal from the dorsally derived cells. The results of our lineage tracing experiments favors the former hypothesis.

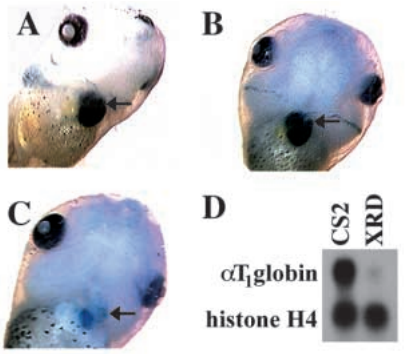

Fig. 7. Benzidine staining of larvae (stage 45-50) injected in the VMZ, arrows point to the heart. (A) Control injected larva. (B) Larva injected with full-length *Xaml* mRNA. (C) Larva injected with *Xrd* mRNA. (D) Northern blot of RNA harvested from stage 38 embryos probed with *α-globin* and *histone H4* (*globin* is reduced 26-fold in the bloodless embryos). Note that this is an earlier stage than is shown in A, B or C.

At later stages, *Xaml* expression in the VBI spreads posteriorly in a wave-like fashion. The results of our explant experiments demonstrate that de novo *Xaml* expression at this stage appears in cells that are ventrally derived. In whole embryos, this aspect of *Xaml* expression precedes and is predictive of future *α-globin* expression. Finally, as *α-globin* expression reaches its posterior boundary of the proctodeum, *Xaml* expression in the VBI begins to decline. This aspect of *Xaml* expression implies a transient requirement for *Xaml* in the early establishment of hematopoietic precursors and suggests *Xaml* is not required for maintenance of cell fate in the primitive erythroid lineage. A similar situation is seen in *Drosophila* where RUNT has an early and transient requirement in cell fate specification during sex determination, segmentation and neurogenesis (Butler et al., 1992; Duffy et al., 1991; Torres and Sanchez, 1992). Based on the observations described above, we propose *Xaml* is a molecular marker of early cell fate specification of the HSC.

Our experiments directly address the origin of *Xaml*-expressing cells at the gastrula stage. We have shown both the dorsal and ventral marginal zones of gastrula stage embryos give rise to *Xaml*-expressing cells in explants. Our lineage tracing and our dominant negative experiments, support the idea that the dorsally derived cells that express *Xaml* populate the anterior VBI and may directly give rise to *α-globin*-expressing cells. It is interesting to compare what we have observed for *Xaml* with previous analyses of HSC origin in amphibians. Our data support early work using transplantation of tissues into cytogenetically labeled hosts, which found an anterior origin of the HSCs that give rise to thymocytes in *Rana pipiens* (Turpen et al., 1973; Volpe and Turpen, 1975), but contradicts other reports claiming that all HSCs originate in the posterior or in the ventral marginal zone (Turpen et al., 1997; Volpe et al., 1979). We believe there are two explanations for the discrepancy between our data and the most recent work by Turpen et al. (1997). First, the dorsally derived *Xaml*-expressing cells originate in the endomesoderm. It is possible that endomesoderm was not included in the DMZ transplants that were used to examine possible dorsal contributions to the VBI (Turpen et al., 1997). Secondly, from our results, the dorsally derived *Xaml* cells are in the anterior region of the embryo at early neurula stages. This region of the embryo would not have been included in the transplants as described by Turpen et al. (1997) as the authors considered the presumptive VBI to be in a centrally located region on the ventral side of the early neurula stage. In fact, our data may clarify early conflicting reports finding a posterior origin of HSCs in one case and an anterior origin of HSCs in another case (Volpe et al., 1979; Volpe and Turpen, 1975) if it is realized the position of HSCs in the embryo is dynamic and composed of *both* anterior cells (dorsally derived) and posterior cells (ventrally derived). The dynamic nature of the

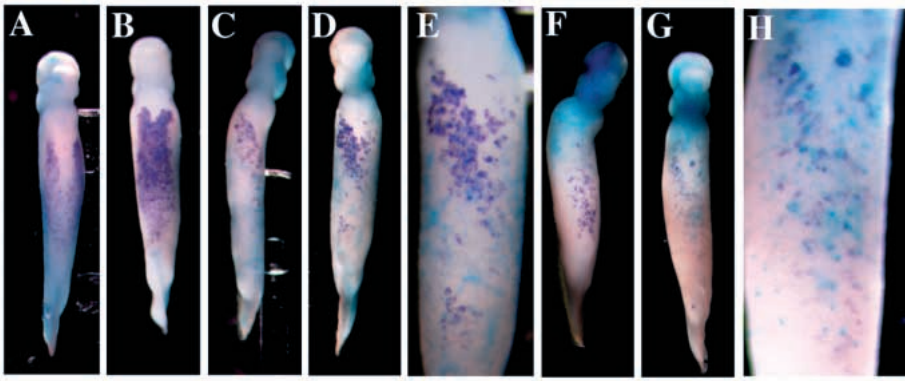

Fig. 8. Embryonic *α-globin* staining in embryos injected with *XRD+lacZ* mRNA (B-H) or *lacZ* alone(A). Blue staining indicates cells that received the *lacZ* message, purple staining indicates cells expressing *α-globin.* (A) embryo injected ventrally with *lacZ* alone, note the significant overlap of blue and purple staining. (B) Embryo injected with *XRD+lacZ* and processed simultaneously with the embryos in C-H; note the normal *α-globin* expression in the VBI and the lack of blue staining in the VBI. (C,D) Embryos injected with *lacZ* staining overlapping the posterior VBI (i.e.. ventrally injected) showing significant inhibition of posterior *α-globin.* (E) Detail of embryo shown in D; note the small patch of *α-globin*-positive cells in the posterior of the VBI demonstrating that this embryo is at a similar developmental stage as that in A. (F,G) Embryos injected with *XRD+lacZ* with anterior targeting of the VBI typically seen with dorsally injected pigmented controls. Note the absence of *α-globin* in the anterior VBI (*n*=19). (H) Detail of embryo shown in G. Note the complimentary pattern of blue and purple staining. The blue cells are in close proximity to purple cells suggesting that XRD acts cell autonomously. In this experiment, we injected 60 embryos with either *lacZ* alone or with *XRD+lacZ*. Of these embryos, 54 injected with *lacZ* alone survived the entire procedure while 51 injected with *XRD+lacZ* survived. All 54 embryos injected with *lacZ* alone showed normal *α-globin* staining. Of the 51 embryos injected with *Xrd+lacZ*, 16 had normal *α-globin* expression and did not show XRD targetting to the VBI. 28 embryos (54%) showed abnormal *α-globin* staining and all of these showed β-gal staining in the VBI. In these experiments, there were an additional seven embryos injected with *Xrd+lacZ* that demonstrated normal *α-globin* staining and also contained β-gal in the VBI. This incomplete penetrance could be due to variations in the effective levels of XRD in these embryos. Consistent with this, we did not observe overlaps with β-gal and *α-globin* when higher levels of *XRD* mRNA (2 ng/blastomere) were injected.

VBI allows for very different results to be obtained depending on the precise stage and tissues examined in a given transplantation experiment.

A requirement for *Xaml* in primitive hematopoiesis contrasts with the finding that *cbfa2* mutations fail to affect primitive hematopoiesis in the mouse. This can be explained in several ways but we favor a model where CBF is required in murine primitive hematopoiesis but another runt domain gene present in mouse provides functional redundancy and covers the requirement of *cbfa2*. The function of this other RDG in *Xenopus* would presumably be inhibited by XRD simultaneously with *Xaml* inhibition. XRD is therefore able to uncover the requirement of runt domain genes in primitive hematopoiesis. In support of this view is the finding that a dominant negative form of *cbfb* causes a delay in the maturation of the primitive erythrocytes (Castilla et al., 1996).

How does the *Xaml* expression pattern relate to definitive hematopoiesis? It has been shown previously that the majority of adult definitive precursors in *Xenopus* arise in the lateral plate mesoderm (Chen and Turpen, 1995; Kau and Turpen, 1983). *Xaml* expression is in isolated cells of the lateral plate mesoderm of stage 25-28 embryos in a region where *α-globin* expression is never seen. It is thus possible that these cells are definitive precursors migrating to their final position in the embryo. In addition, five to ten cells that express *Xaml* but do not express *α-globin* are found anterior to the VBI in the heart primordium. These cells may be HSCs set aside for later hematopoiesis. Finally, it is also possible a subset of the *Xaml*-expressing cells in the VBI are themselves definitive precursors as cell marking experiments in *Xenopus* have shown a minor contribution of the VBI to adult blood (Smith et al., 1989).

Our results demonstrate the embryological origin of blood in the *Xenopus* embryo is more complex than previously thought. The fact that *Xaml* expression is predictive of future *α-globin* suggests that *Xaml* is an early molecular marker for the HSC. The cells that express *Xaml* but do not express *α-globin* may be HSCs capable of populating other blood compartments at later stages. If this is the case, *Xaml* will prove to be a useful tool for the study of the early embryonic patterning events that lead to the specification of hematopoietic stem cells.

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