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 CBFα 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). Targeted mutations in transgenic mice demonstrate that the *cbfa2* and the *cbfb* genes 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 SmaI and BglIII, 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'-GGAATTCACCATGGTGAAAGATTG-3' and the downstream primer 5'-GCTCTAGACGTCCGTGCGCTGAGG-3'. Following amplification the Runt domain DNA was subcloned into an EcoRI/XbaI-digested pCS2+ expression vector to generate pXRD-8. To generate capped mRNA, pXRD-8 was linearized with PvuII and transcribed in vitro using the SP6 mMessage mMachine™ (Ambion). For functional analyses, full-length Xaml RNA was subcloned into pCS+2 to generate pCSX. The Xaml mRNA for injecting embryos was prepared by linearizing pCSX with NotI 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 SalI followed by in vitro transcription with T7 polymerase. The antisense αTglobin 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 MgSO₄, 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 saturated, the embryos were stored in 100% ethanol until use. The blue color was in 0.1 M phosphate buffer + 0.01 M potassium ferricyanide + 0.01 M formaldehyde + 0.1% X-gal. After the blue color was saturated, the embryos were stored in 100% ethanol until use.

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 αTglobin 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 \( \alpha \)-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 \( \alpha \)-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 \( \alpha \)-globin-expressing cells in similarly staged embryos (Fig. 2G,H). This more posterior Xaml expression is predictive of future \( \alpha \)-globin expression at later stages and continues to expand as the embryos age until it reaches its' posterior boundary at the proctodeum and continues to expand as 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).

**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.

The expression of Xaml in the VBI in a pattern that anticipates the pattern of \( \alpha \)-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.
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 excited 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).
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 ae-globin suggested this question required further investigation.

Surprisingly, we found that treatment of embryos with LiCl affected ae-globin expression in a manner that exactly paralleled that of Xaml expression. Highly dorsalized embryos had greatly reduced ae-globin expression while embryos with intermediate dorsalization showed high levels of ae-globin expression (Fig. 5E). Intense ae-globin expression is seen in the ventral-most region of the DAI 8 embryo. The ae-globin expression domain overlaps with the Xaml expression domain but there are a large number of Xaml-expressing cells anterior to the region that expresses ae-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 ae-globin expression.
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 explant 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 δT-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 ventalization 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 immunohistochemically (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 larve 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 larve (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 posteriorly, 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 postero 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. (E,F) 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 targeting 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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REFERENCES


