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Expression and function of *blimp1/krox*, an alternatively transcribed regulatory gene of the sea urchin endomesoderm network

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Abstract

The *blimp1/krox* gene of *Strongylocentrotus purpuratus*, formerly *krox1*, encodes zinc finger transcription factors which play a central role in both early and late endomesoderm specification. Here we show that there are two alternative splice forms transcribed under the control of different regulatory regions. The *blimp1/krox1b* form was previously unknown, and is the form expressed during cleavage, beginning 6–9 h postfertilization. This form is required for the early events of endomesoderm specification. A different splice variant, *blimp1/krox1a*, is expressed only from gastrula stage onward. During cleavage stages the *blimp1/krox* gene is expressed in the large micromeres and veg2 descendents. Soon after, it is expressed in the ring of specified mesoderm cells at the vegetal pole of the blastula. Its expression is later restricted to the blastopore region and the posterior of the invaginating archenteron, and finally to the midgut and hindgut of the pluteus larva. The expression of *blimp1/krox* is dynamic, and involves several distinct spatial territories. A GFP recombinant BAC was created by substituting the GFP coding sequence for that of the second exon (1b), in order to distinguish the expression pattern of the early form from that of the late form. This construct closely mimics *blimp1/krox1b* expression during early stages of sea urchin development. To expand our knowledge of the downstream linkages of this gene, additional experiments were carried out using antisense morpholino oligos (MASO). We confirmed previously published data that *blimp1/krox* autoregulates its own expression, but discovered, surprisingly, that this gene represses rather than activates itself. This negative autoregulation is restricted to the mesodermal and probably skeletogenic territories during the blastula stage, as shown by in situ hybridization analysis of MASO injected embryos. The MASO perturbation analysis also revealed *blimp1/krox* inputs into other genes of the endomesoderm regulatory network.

JEL classification: blimp1/krox; *krox1*; *blimp1*; *blimp-1*; *blmp-1*; *prdm1*; *prdi-bf1*; *znfpr1a1*; *ubo*; *odd-3*
Keywords: Strongylocentrotus purpuratus; SET domain; PR domain; Cys2His2 zinc finger transcription factor; Endomesodermal specification; Gene regulatory network; Alternative splice forms

Introduction

In sea urchins, territorial specification is initiated early in cleavage. The endomesoderm lineages originate from the vegetal half of the embryo. This process involves maternal determinants segregated to this area as well as intercellular signaling interactions (Davidson, 1986, 1989; for recent reviews Ransick and Davidson, 1998, 2001; Angerer and Angerer, 2003). General understanding of the transcriptional control of endomesoderm specification in the sea urchin embryo is summarized in a gene regulatory network (EM-GRN) connecting more than 40 regulatory genes that are expressed between egg and late blastula stage embryos (Davidson et al., 2002a,b; Oliveri and Davidson, 2004; Levine and Davidson, 2005). Individual genes encoding regulatory factors have been linked into the GRN by means of experimental perturbations in which expression of the gene is either knocked down or its product is replaced with a dominant negative form. The linkages between genes indicated in the GRN provide predictions of the *cis*-regulatory interactions that drive the process. These predictions are tested through *cis*-regulatory analysis of functional elements capable of generating expression in the

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correct spatial and temporal domains (for current review, Levine and Davidson, 2005; a continually updated version of the network, and supporting data, are at http://sugp.caltech.edu/endomes/).

Among the early zygotic regulatory genes required for specification of the sea urchin embryo endomesoderm is the *blimp1/krox* gene. This gene (initially called *krox1*) was cloned from a gastrula stage cDNA library in a screen for zinc finger transcription factors, and was reported to be transiently expressed in the vegetal plate territory (Wang et al., 1996). Its encoded protein is most similar to B-lymphocyte-induced maturation protein 1 (Blimp-1) in vertebrates, a transcriptional regulator of B-cells (Chang and Calame, 2002; Chang et al., 2000; Siammas and Davis, 2004). Here we rename the gene *blimp1/krox*, as it is in fact not a member of the *krüppel* gene family, as originally claimed. The *blimp1/krox* factor is a member of the SET domain family of proteins that recruits methyltransferases, which may directly modify histones (Jenuwein, 2001). "SET" stands for *S*u(var)3–9, *E*nhancer-of-zeste, and *T*rithorax, all proteins which contain this functional domain. The structure of the SET domain has been established in yeast to have an overall fold rich in beta-strands, a potential active site consisting of a SAM binding pocket, and a connected groove that could accommodate the binding of the N-terminal tail of histone H3 (Min et al., 2002). The "PR" subset of SET domains, the one present in Blimp1 family members, is somewhat divergent. It mediates protein–protein interactions, and its orthologue in mice (synonyms: Prdm1, and PrdI-bf) has been shown to have a modular structure, such that particular domains are required for the regulation of subsets of its downstream target genes (Siammas and Davis, 2004). In humans, the PrdI–Bf1 (Blimp1) protein products act as transcriptional repressors in myeloid cells and recruit methyltransferases to promoter sites where they induce histone H3 methylation (Györy et al., 2003, 2004). *blimp1* null (−/−) mice die during early embryogenesis, as this gene plays an important role in gastrulation. Conditional knock-outs in B-cell lines show that it is essential for their differentiation into plasma cells (Shaffer et al., 2004; Siammas and Davis, 2004). In mouse, it is the balance of expression between the two alternatively spliced isoforms, one of which is missing the PR domain, which regulates cellular proliferation and differentiation (Gyory et al., 2003). Mouse *blimp-1* is also required for germ cell development (Ohinata et al., 2005). In chicks, this gene is expressed in the apical ectodermal ridge and posterior dorsal ectoderm of developing limb buds (Ha and Riddle, 2003). *Xenopus blimp1* has an important role in endomesoderm specification, acting to promote anterior endoderm development and spatially restricting mesoderm formation (de Souza et al., 1999). Recently, the zebrafish orthologue of *blimp-1*, also called *ubo*, has been shown to be important for gastrulation, muscle specification, and neural crest development (Baxendale et al., 2004; Roy and Ng, 2004). The *blimp-1* gene is essential for slow twitch muscle fiber specification, and besides repressing fast MyHC, it also acts as a positive activator of the slow MyHC isoform and Prox1 proteins (Baxendale et al., 2004). Thus, this gene has many different functions, a feature which, as we shall see, it displays in sea urchin embryos as well.

As previously reported, injection of mRNA into sea urchin eggs encoding the DNA binding domain of *blimp1/krox* fused to the repressor domain of the *Drosophila* Engrailed factor revealed some of its downstream target genes in the EM-GRN (Davidson et al., 2002a,b). We showed that *blimp1/krox* is necessary for initiation and maintenance of the expression of *otx* in the endomesoderm, and this was subsequently confirmed to be a direct *cis*-regulatory function (Yuh et al., 2004). It is also important for the specification of endodermal cells from the veg1 tier where it regulates *eve* and *hox11/13b*. In the present work, we demonstrate that the *blimp1/krox* gene produces splice isoforms that are alternatively transcribed, and alternative splicing is a conserved feature of this gene in deuterostomes. The two isoforms are expressed in a different spatial–temporal pattern. The early form is *blimp1/krox1b*, and this is the form present during endomesoderm specification in the period modeled by the EM-GRN. Expression of the late form, *blimp1/krox1a*, begins only in the early gastrula. Therefore the *cis*-regulatory control system operating the *1b* transcription unit is that relevant to the EM-GRN.

Materials and methods

5′ Race and sequencing

A 10-h postfertilization (hpf) race library made using the GeneRacer Kit (Invitrogen, Carlsbad, CA) was used to extend the message sequence further 5′ of the known *blimp1/krox* mRNA. A primer in exon 2 (*blimp1/krox* race R: 5′-TGTCAGACGGCACGGCGTTGTCGTTGCA-3′) was used. The resulting fragments were subcloned into a TA cloning vector (pGEMTeasy, Invitrogen, Carlsbad, CA) and sequenced in an ABI 377 sequencer using ABI Prism BigDye Terminator Cycle Sequencing (Applied Biosystems, Foster City, CA). The resulting sequences were blasted against the *blimp1/krox* cDNA and *Spblimp1/krox* BAC sequences using the BLAST feature in Family Relations (Brown et al., 2002, 2005).

Embryo handling

Adult *Strongylocentrotus purpuratus* (*S. purpuratus*) animals were maintained at the Caltech Marine Biological Laboratory, and gametes were shed according to standard procedures (Leahy, 1986). Embryos were obtained, cultured, collected and microinjected as previously described (Foltz et al., 2004; Cheers and Ettensohn, 2004) with minor modifications. All injection solutions contained 15% glycerol and 0.12M KCl, and were injected into fertilized eggs using a Picospritzer (Parker Instrumentation, Fairfield, NJ). When injecting, we attempted to cause a clearing in the egg cytoplasm of 1/5 to 1/4 of the egg diameter corresponding to a 2- to 4-pl injection volume.

Morpholino oligonucleotide sequences

The sequence of the anti-*blimp1/krox1b* MASO which is targeted against the translation initiation site of the early form is 5′-CTCCCTTTCGCTTGAAAAA-CACCGC-3′ (complementary to nucleotide positions −27 to −3 with respect to the translational start site of *blimp1/krox1b* mRNA). We injected 2 to 4 pl at a concentration of 100 μM of this morpholino when indicated, in conjunction with a MASO targeting the late form, anti-*blimp1/krox1a* M1: 5′-AGACGG-CACGGCGTTGTCGTTGCAC-3′ (nt position +6 to +31 of *blimp1/krox1a*

mRNA) or Anti-*blimp1/krox1a* M2: 5′-CGGCGTTGTCGTTGCACCCCATCGC-3′ (nt position −3 to +22 of *blimp1/krox1a* mRNA) at 200 μM concentrations. In all experiments, as a negative control, embryos injected with 300 μM of the standard control morpholino (SCM) were included. All morpholinos were acquired from Gene Tools (Corvallis, OR).

blimp1/krox knockdown perturbation

Morpholino oligos were resuspended in water to a concentration of 500 μM or 1 mM. A working solution of 100 to 300 μM of morpholino oligos in 15% glycerol and 0.12 M KCl was injected into fertilized eggs. The standard control morpholino (SCM) from GeneTools (Corvallis, OR) was used at equal or greater concentration as a control in every experiment and compared side by side with uninjected and MASO embryos (Angerer and Angerer, 2004). The efficacy of the anti-*blimp1/krox1a* and anti-*blimp1/krox1b* morpholinos was assessed in initial experiments through co-injection with GFP mRNA containing sequence complementary to the respective morpholino (data not shown).

BAC recovery and sequencing

Spblimp1/krox (clone 163O19) and *Lvblimp1/krox* (clone 60B16) BACs were obtained by hybridization of a *Spblimp1/krox* cDNA fragment to arrayed genomic BAC libraries for *S. purpuratus* and *Lytechinus variegatus* (*L. variegatus*) respectively (Cameron et al., 2000, 2004). *Spblimp1/krox* and *Lvblimp1/krox* BACs were sequenced by the DOE's Joint Genome Institute (Genebank accession nos. AC131508; AC131502).

BAC sequence annotation

The BAC sequences were annotated using the Sea Urchin Genome Annotation Resource (SUGAR), as well as Family Relations (Brown et al., 2002, 2005).

BAC homologous recombination

Using the homologous recombination machinery from bacterial cells, the sequence coding for exon1b from *Spblimp1/krox* BAC 163o19 was substituted for that of green fluorescent protein (Yu et al., 2000). To create the cassette containing GFP and kanamycin with flanking regions homologous to the BAC, a PCR approach was taken. Briefly, 45 bp of sequence homologous to the BAC on the 5′ end of the region to be recombined is attached to the 5′ end of the GFP primer (Sp_*blimp1/krox*_(1b)right: 5′CTGCCCCATTCATCACATTTTCAACAATCTGAGTCGACAGATGACTCGAAGAGCTATTCCAGAAGTAGTGA-3′) such that it is added to the product when the primer is used to amplify the construct containing the GFP/Km cassette. In the same manner, 45 bp of sequence homologous to the 3′ end of the region to be flipped out is attached to the 5′ end of the kanamycin primer (Sp_*blimp1/krox*_(1b)left: 5′ TTGTTGTGATTTTGTACCGCGGTGTTTTTCAAGCGAAAGGGAGAAATGAGCAAGGGCGAGGAACT-3′). After PCR amplification using the two primers containing the homologous sequence tails and the construct containing the cassette as the template, the product was purified using MiniElute PCR Purification Kit (Qiagen, Valencia, CA), and subsequently digested with *Dpn*I (New England Biolabs, Ipswich, MA) to remove traces of the original cassette. This fragment was used to transform competent EL250 cells containing the BAC construct, and the kanamicyn gene was removed as previously described (Yu et al., 2000). Recombinant BACs were screened by sizing the inserts using PCR and subsequently sequenced using outside flanking primers (Out-Sp1b-F: 5′-CTCATCTACTTTCGCTGCCAGTACT-3′, and Out-Sp1b-R: 5′-CTCATTATAGTTGATGGACATACTCATATC-3′).

Recombinant GFP-BAC transgenesis

Spblimp1/krox1b-GFP BAC was purified using Maxi NucleoBond® Plasmid Kit according to instructions from the manufacturer (Clontech, Mountain View, CA). After linearizing using *Asc*I (New England Biolabs, Ipswich, MA), the digested BAC was loaded onto a CL4b Sepharose column (Pharmacia, Uppsala, Sweden) and fractionated into small aliquots (Hammes and Schedl, 2001). Optical densities were taken from each fraction at 260 and 280, and the first to contain a significant amount of DNA is used for microinjection. The size and quality of the fractions were accessed by pulse filed gel electrophoresis. The injection solution contained 500 molecules per 2 pl of *Spblimp1/krox1b*-GFP BAC. Embryos were injected as described by Rast (2000) with minor modifications. No carrier DNA was added as BACs are long enough not to require it for linear incorporation, and a final concentration of 15% glycerol with 0.12 M KCl was used.

WMISH probes

Digoxigenin-labeled RNA probes were made as previously described (Yuh et al., 2002). Briefly, gene fragments were amplified by PCR and subcloned into and TA cloning vector (Invitrogen, Carlsbad, CA). Constructs were linearized 3′ of the probe sequence in relation to the transcriptase promoter used. All probes corresponded to the antisense as well as the sense direction. No staining was observed using the sense probes (Fig. 12, see Supplemental Materials in online version of this article). *blimp1/krox* Probe Primer F: 5′-TTCTTCCGATCACCTTGCTG-3′, and *blimp1/krox* Probe Primer R: 5′-GAAAGATAGCCATTGGAATCTGC-3′.

WMISH

Whole mount in situ hybridizations were performed as previously described (Minokawa et al., 2004) with minor modifications. Embryos were collected at different developmental stages and fixed in 4% paraformaldehyde, 32.5% filtered seawater, 32.5 mM MOPS (pH 0.7), and 162.5 mM NaCl. When looking at endogenous message distribution, a hybridization buffer containing 70% formamide was used. When looking at message generate from a transgene, such as GFP, the hybridization buffer contained 50% formamide. In both cases the embryos were hybridized for 5 to 8 days at 48°C with occasional mixing. An additional high temperature wash in MOPS buffer was added after the high temperature wash in hybridization buffer. Embryos were mounted in 50% glycerol, visualized using Nomarsky optics, and imaged with a color digital camera. Images were collected and processed using Adobe Photoshop.

QPCR

Temporal accumulation of messages was monitored using real time quantitative polymerase chain reaction (QPCR). Approximately 500 embryos from different stages were collected. RNA was isolated using RNAeasy micro kit (Qiagen, Valencia, CA), and DNase treated using DNA-free kit (Ambion, Austin, TX) according to instructions from the manufacturer. Reverse transcription into cDNA was performed using Taqman Gold RT kit following instructions from the manufacturer (Applied Biosystems, Foster City, CA). Quantitation of the mRNA was performed as described by Oliveri et al. (2002).

The expression of putative downstream targets of *blimp1/krox* was monitored by QPCR. Either 100 or 200 injected embryos were collected for RNA isolation into RNA-Bee (Leedo Medical Laboratories, Houston TX). Reverse transcription into cDNA was performed using Taqman Gold RT kit following instructions from the manufacturer (Applied Biosystems, Foster City, CA). Fold changes in expression in control and morpholino injected embryos was calculated as previously described (Davidson et al., 2002a,b).

Briefly, the equivalent of two embryos was used as template in each reaction in the presence of 5 pmol of each primer (forward and reverse). Primer sequences can be found at http://sugp.caltech.edu/resources/methods/q-pcr.psp or are listed below. SYBR Green was used to monitor product accumulation in real time and ROX was used as a measure of background fluorescence in a 7900 (Applied Biosystems, Foster City, CA) as previously described (Rast et al., 2000; Ransick et al., 2002).

The very short length of the 1a and 1b exons (116 bp, and 226 bp, respectively) made finding appropriate primer pairs that would amplify only one or the other splice form difficult. For this reason we used more than one set of primers. Three independent oligo primer pairs were synthesized to amplify

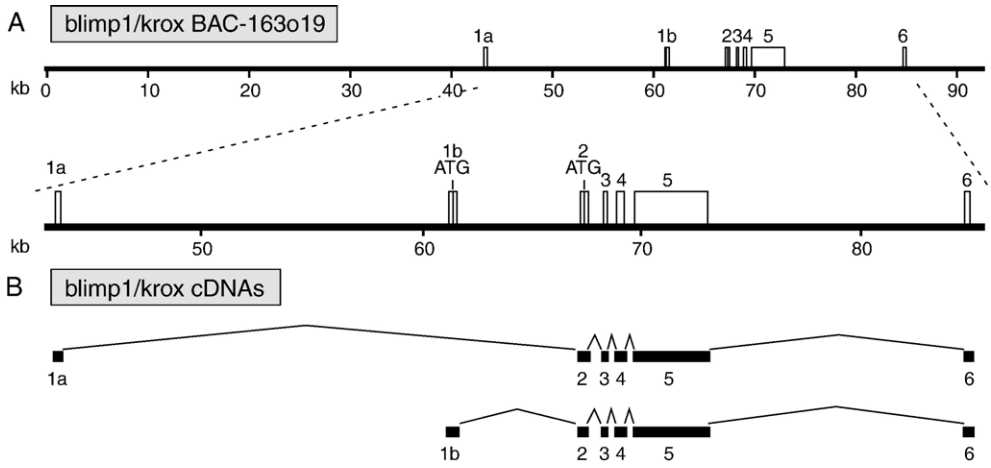


Fig. 1. Structure of *blimp1/krox* gene and splicing isoforms. (A) Annotation of BAC 169o19 showing the position of *blimp1/krox* exons. The antisense morpholino against the early form binds in the region surrounding the ATG in exon 1b, while the late form morpholino binds in the region surrounding the ATG in exon 2. Numbered boxes represent the location of exon sequences on BAC. (B) Structure of the alternatively transcribed and spliced cDNAs. In the *blimp1/krox1a* transcript, exon 1a is spliced to exon 2. Thus, the late form is (exon 1a + 2 + 3 + 4 + 5 + 6). In the *blimp1/krox1b* transcript, exon 1b is spliced to exon 2; thus the early form is (exon 1b + 2 + 3 + 4 + 5 + 6). The *blimp1/krox1a* form is that described previously (as *Spkrox1*; Wang et al., 1996).

blimp1/krox1a message, and two independent oligo primer pairs were designed for *blimp1/krox1b*. They were utilized in QPCR experiments to measure the transcripts present in sea urchin embryos over time.

Sp*blimp1/krox* exon1a only F: 5′-AAGCACTTGCTTGCTGTTACC-3′
Sp*blimp1/krox* exon1a only R: 5′-AAAATAGCTTGCGGTTTCAATC-3′
Sp*blimp1/krox* exon1a + 2 F1: 5′-GGAAAGCACTTGCTTGCTGT-3′
Sp*blimp1/krox* exon1a + 2 R1: 5′-CGAAGACCTGATCGAAGACC-3′
Sp*blimp1/krox* exon1a + 2 F2: 5′-CGATTGAAACCGCAAGCTAT-3′
Sp*blimp1/krox* exon1a + 2 R2: 5′-ATCGACCTCGGTCATGTCA-3′
Sp*blimp1/krox* exon1b only F: 5′-GCGAGGGTGTTCAACGATA-3′
Sp*blimp1/krox* exon1b only R: 5′-TCAAGGATAGCGGACACTCA-3′
Sp*blimp1/krox* exon1b + 2 F: 5′-CTAGCAATGCGGGATCTCTACT-3′
Sp*blimp1/krox* exon1b + 2 R: 5′-CGAAGACCTGATCGAAGACC-3′

Protein sequence alignment and phylogenetic tree

Nucleotide sequences were translated using the Expasy-translate tool (http://us.expasy.org/tools/dna.html). Available protein sequences (as indicated below) were aligned using ClustalX version 1.81 (Thompson et al., 1997). Alignment output file was formatted using Boxshade (http://www.ch.embnet.org/software/BOX_form.html). A neighbor-joining tree was constructed using MEGA version 2.1 or 3 (Kumar et al., 2001, 2003) and tested by bootstrapping using default parameters. Protein domains were mapped using InterProScan (http://www.ebi.ac.uk/InterProScan/).

Species abbreviations: Sp = *Strongylocentrotus purpuratus*; Lv = *Lytechinus variegatus*; Am1 = *Asterina miniata*; Tn = *Tetraodon nigroviridis*; Fr = *Fugu rubripes (Takifugu rubripes)*; Dr = *Danio rerio*; Xl = *Xenopus laevis*; Gg = *Gallus gallus*; Mm = *Mus musculus*; Rn = *Rattus norvegicus*; Pt = Pan troglodytes; *Hs* = Homo sapiens; Ce = *Caenorhabditis elegans*; Cb = *Caenorhabditis briggsae*; Dm = *Drosophila melanogaster*; Ag = *Anopheles gambiae*; Am2 = *Apis mellifera*.

Accession Numbers for sequences used in alignment: GeneBank accession nos. DQ225099, DQ177152, AY196329, AY196905, AY497217, CAG11080, AB126229, AF182280, AC147720, AF305534S6, XM_228320, XM_518658, AF084199, Z78418, CAE58934, AY071225, XM_391847, XP_316619.

Spblimp1/krox1a: DQ225099; *Spblimp1/krox1b*: DQ177152; *Amblimp/krox-alpha*: AY196329; *Amblimp/krox-beta*: AY196905; *Drblimp1*: AY497217; *Tnblimp1:* CAG11080; *Frblimp1*: AB126229; *Xlblimp1*: AF182280; *Ggblimp1:* AC147720; *Mmblimp1*: AF305534S6; *Rnblimp1*: XM_228320; *Ptblimp1*: XM_518658; *Hsblimp1*: AF084199; *Ceblmp-1*: Z78418; *Cbblmp-1*, CAE58934; *Dmblimp-1*: AY071225, *Amblimp-1*: XM_391847, *Agblimp-1*: XP_316619.

Diagrams, graphs and line drawings

Figures were made using Adobe Illustrator CS or Adobe Photoshop CS. Gene network diagrams were made using BioTapestry version 2.1 (Longabaugh et al., 2005). Temporal expression graph was drawn using GraphPad Prism 4.

Results

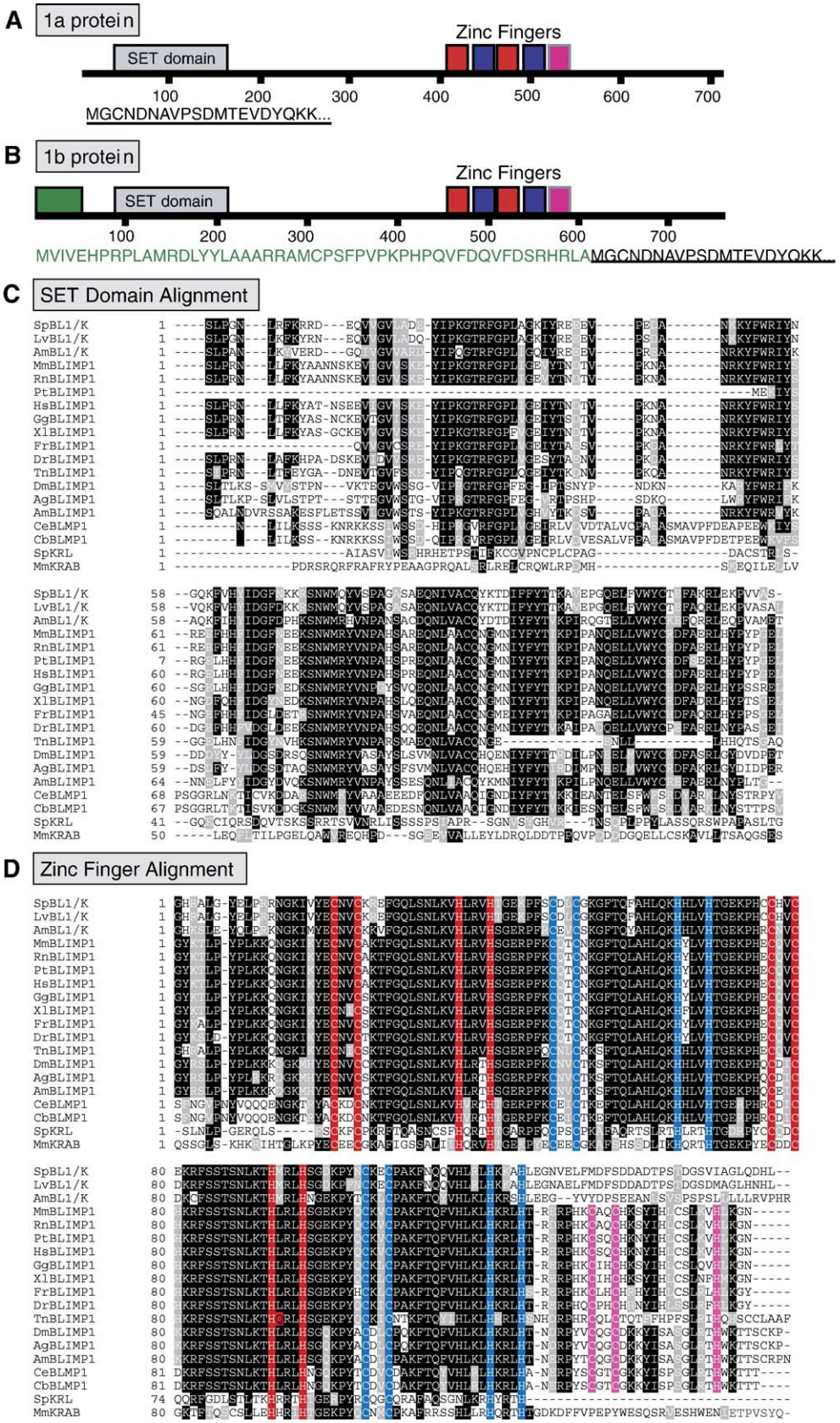
Gene structure and isolation of early splice form

The *blimp1/krox* locus is 42 kbp and the gene is split into seven exons (Figs. 1A, B). A nucleotide alignment between the BAC sequence and cDNA sequences can be found in Supplemental Materials. Exons 1a and 6 contain only nontranslated sequence, and the 3′ UTR is quite long. The genomic organization of the locus is conserved between *S. purpuratus* and *L. variegatus* (data not shown). There are no other genes predicted within this region.

Fragments obtained from a 5′ race library contained a novel sequence that aligned to the *blimp1/krox* BAC genomic sequence between the previously known *blimp1/krox* exon 1

Fig. 2. *blimp1/krox* protein isoform domains. (A) 1a (late) form; (B) 1b (early) form. Four complete Cys2His2 Zn fingers and one degenerate Zn finger are indicated by colored boxes. The N-terminal amino acid sequences are shown beneath; the first 50 amino acids are unique to the 1b protein (green). (C) SET domain alignment (see Materials and methods for abbreviations and sources). All orthologues of *blimp1/krox* contain an N-terminal SET domain, mediating protein–protein interaction, and possibly conferring protein–methylating enzyme activity. (D) Zn finger alignment. The degenerate 5th zinc finger no longer follows the consensus sequence, but can easily be identified. Global alignment including isoform sequences can be seen in Supplemental Materials. Colored bars, coded as in (A) and (B) denote the individual Zn fingers. Cysteins and histidines forming the C2H2 zinc finger structure are highlighted.

and exon 2 (see Fig. 1 of Supplemental Materials). We named the 5′ most exon of *blimp1/krox* "1a" and the following one "1b," as they are alternatively used. The *blimp1/krox1b* form was found to be an alternative splice form by QPCR amplification of cDNAs from different embryonic stages, using primers that would recognize this new sequence. This



exon contains a 5′ UTR as well as coding sequence for 50 extra amino acids when spliced to exon 2 (Fig. 2B and Supplemental Materials). Therefore this isoform utilizes a different translation initiation sequence from that of the other splice form, in which the first exon contains only 5′ UTR (Figs. 1C and 2A). There are three ATGs in frame within exon 1b that could act as translation initiation sites (Fig. 1B), but only the most upstream one has a consensus Kozac sequence. As the predicted amino acid sequence from *L. variegatus* also corresponds to this longer form, we assume that the upstream most ATG, yielding the longer peptide, is the one utilized. Exons 1a and 1b utilize different transcription initiation sites, and as will be described elsewhere, distinct regulatory apparatus. All other exons are included in both of the splice forms (sequence from exon 2 was part of the isolated fragment from the race library). The newly described exon 1b is highly conserved between *S. purpuratus* and *L. variegatus* (full alignment is shown in Supplemental Materials; *L. variegatus* diverged from *S. purpuratus* approximately 50 mya; Smith, 1988; Lee, 2003). *Asterina miniata* (*A. miniata*), a starfish, likewise has two similar alternative splice forms (Hinman and Davidson, 2003). However, the additional N-terminal amino acids encoded by sea urchin exon 1b are not well conserved in starfish (Supplemental Materials).

The proteins encoded by the *blimp1/krox* are 703 and 753 amino acids long, and correspond to the late and early forms respectively (Figs. 2A, B). The translation is different from that previously published on the basis of a cDNA clone (Wang et al., 1996) due to a stop codon in the BAC sequence in the absence of which the peptide would be 837 amino acids in length. It is unclear if this difference is the result of a polymorphism in the population, a sequencing error, or a mutation in the clone isolated. All recognized protein domains are present using either translation. The protein includes classic Cys2His2 (C2H2) zinc fingers which are characterized by the sequence C(X)2–4,C(X)8, H(X)3–5, H (Evans and Hollenberg, 1988).

Phylogenetic analysis

The multiple alignment of Fig. 2C and the phylogenetic analysis of Fig. 3 indicate that sea urchin *blimp1/krox* is indeed the orthologue of the vertebrate *blimp1/prdm1/prdI-bf1* genes. The two most prominent domains present in the *blimp1/krox* protein are the SET domain (more specifically a PR domain), and the four DNA-binding C2H2 zinc fingers followed by a fifth divergent zinc finger. As shown in Figs. 2C, D, both of these domains are highly conserved (Figs. 2C, D, 3, and Supplemental Materials).

The taxonomic distribution that can be seen in the protein sequence tree matches what would be expected from a clade-built tree. The ecdysozoan proteins all group together as do the deuterostome Blimp1 proteins. If only the zinc fingers are used to build the tree, the relationship between the groups does not hold and many groupings appear polyphyletic. All three echinoderm sequences (i.e., *Strongylocentrotus*, *Lytechinus*, and *Asterina*) are more closely related to the vertebrate sequences than to the ecdysozoan sequences, thereby forming a monophyletic deuterostome group.

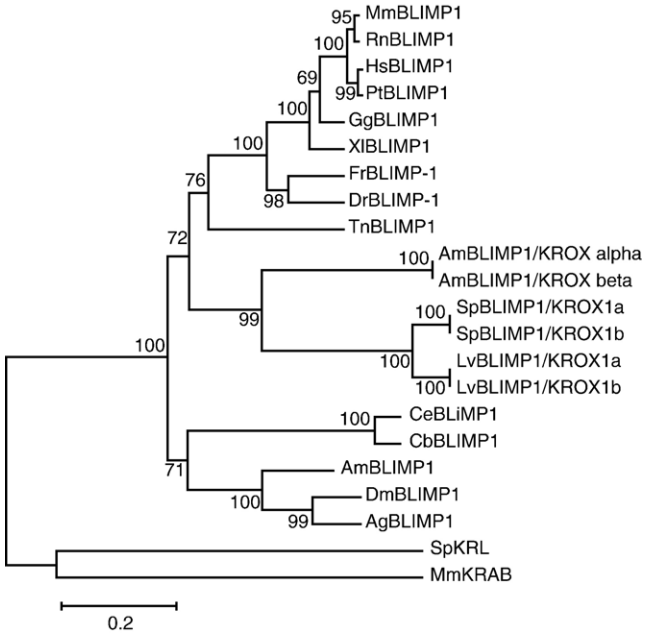


Fig. 3. Phylogenetic tree of the Blimp1 gene family. A neighbor-joining tree was constructed using MEGA version 2 (Kumar et al., 2001) and tested by bootstrapping using 1000 replicates to infer the reliability of branching points. The scale bar represents the number of amino acid substitutions per site, and is based on the amino acid sequences with Poisson corrected distances. The tree is based on a multiple alignment shown in Supplemental Materials Fig. 2.

Temporal expression of the alternative splice forms

We looked at the temporal expression pattern of the two splice forms by QPCR, utilizing primers that distinguish them (see Material and Methods for details). The time-course of accumulation of their respective transcripts is shown in Fig. 4. The blue line represents the expression of the early form, *blimp1/krox1b*. This message can first be detected between 6 and 9 hpf (cleavage stage), and its expression peaks at 42 hpf (late gastrula stage) at around 7500 molecules per embryo. There are about 60 cells of the endoderm in the late blastula and early gastrula, so there are about 125 molecules per cell of transcript from the early transcription unit. Thereafter, it rapidly declines. Thus *blimp1/krox1b* is the isoform expressed during the time period when the endomesoderm territory is being specified, from soon after the birth of the large micromeres to gastrulation.

The green line represents the expression of *blimp1/krox1a*, the late form. It is not expressed until sometime between 30 and 36 hpf, and its transcription persists past 84 hpf into the late pluteus stage. It is expressed at the highest levels between 54 and 72 hpf, accumulating around 1500 molecules per embryo. At this time, there are approximately 60 midgut and 60 hindgut cells bringing the expression of *Spblimp1/krox1a* to 12 molecules per average cell. Thus, *blimp1/krox1b* is expressed at about a 5-fold higher level per embryo, comparing respective peak expression levels, than is *blimp1/krox1a*.

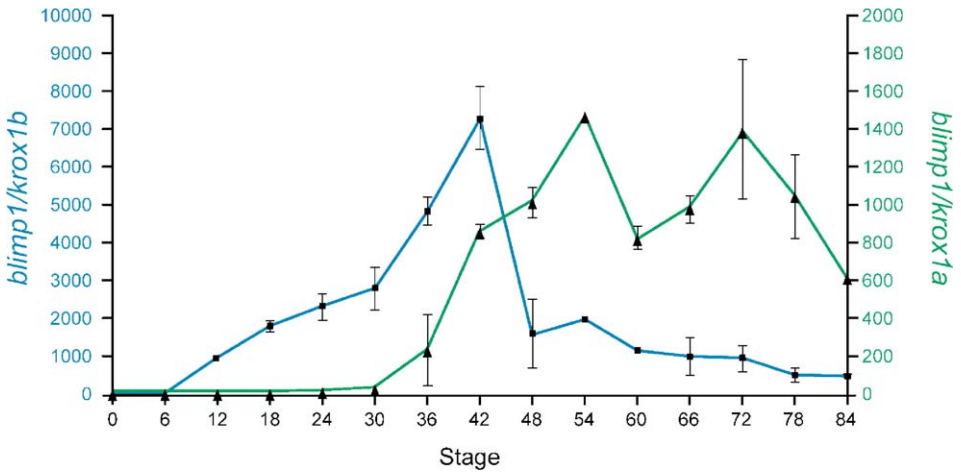


Fig. 4. Temporal expression of endogenous *blimp1/krox* gene. Quantitative real time PCR measurements show levels of expression of *blimp1/krox1a* and *blimp1/krox1b* mRNAs at different developmental stages. Results were normalized to levels of the transcript of the *Z12-1* gene, the abundance of which remains relatively constant though out the stages monitored, in order to obtain the number of molecules per embryo (Oliveri et al., 2002). Two independent primer sets were used to measure expression of *blimp1/krox1b*, and three independent primer sets were used to measure *blimp1/krox1a*. Error bars represent one standard deviation. *blimp1/krox1b* begins to be being expressed after 6 hpf (some time points are not shown in graph). *blimp1/krox1a* starts being expressed after 30 hpf.

Spatial expression of the blimp1/krox gene

The *blimp1/krox* gene is expressed in multiple tiers of cells in the vegetal plate, including the large micromeres and the veg2 and veg1 lineages, at different stages of development. A whole mount in situ hybridization series is displayed in Fig. 5A. Between 6 and 9 h postfertilization *blimp1/krox* mRNA accumulates in the large micromeres, but this gene is not expressed in the small micromeres, nor in any of their descendants during embryogenesis (note unstained vegetal-most cells in the 10 h vegetal view (VV) of Fig. 5A). By 10 hpf, it is also expressed in the veg2 tier of endomesodermal precursors, but its expression disappears from the micromeres soon after this time, as can be seen in the 18-h VV of Fig. 5A. It has been cleared from the mesodermal lineages of these territories by a few hours later, when it also begins to be expressed in a new territory, a subset of veg1 tier descendents which will become part of the gut (21 and 25 h embryos in VV, Fig. 5A). Expression is strongest in the blastopore region of the early and mid gastrula (36 and 48 h embryos, Fig. 5A), and encompasses the midgut as well as the hindgut of the later gastrula and larva (72 h embryo, Fig. 5A). Midgut expression is very likely activated by Brn1/2/4, as anti-*brn1/2/4* morpholino antisense oligo (MASO) down-regulates *blimp1/krox* expression at 36 hpf (Yuh et al., 2005), and *brn1/2/4* is expressed at the right time and place.

A diagrammatic summary of *blimp1/krox* expression throughout the whole course of development is shown in Fig. 5B, as viewed from the side of the embryo; and in Fig. 5C, the pattern of expression is portrayed as seen from the vegetal pole for the cleavage–blastula period. From here, the dynamic distribution pattern of *blimp1/krox* message is most obvious. This gene is not only activated in different lineages, but in the skeletogenic and mesodermal territories, it is also repressed in successive concentric domains some hours after its activation.

Expression of a blimp1/krox1b-GFP knock-in BAC

According to the measurements in Fig. 4, transcripts accumulated before 30 hpf are solely the product of the early 1b transcription unit. Nonetheless, to provide an independent indication of the spatial expression of the 1b regulatory system, we created a GFP knock-in (Yu et al., 2000) that would specifically report the activity of the early transcription unit. Thus, the GFP coding sequence was inserted in place of the exon1b coding sequence, immediately following the ATG start codon (Fig. 6A). The expression of the transgene was monitored by GFP WMISH, so that the location of the stain would indicate the contemporary expression domain rather than the accumulation of the long-lived GFP protein. Fig. 6B shows examples of WMISH embryos injected with *Spblimp1/krox1b-GFP* BAC in side view. Stages are indicated in the top right hand corner. It can be seen that in these examples, the reporter construct has been incorporated in one half or one fourth of the embryo, which is not infrequently seen with injected BACs (S. Damle and E. Davidson, unpublished results). At 17 hpf, GFP message is found throughout the vegetal plate, i.e., in micromere as well as veg2 lineages (top row in Fig. 6B), while at 24 hpf, the signal is present in both veg2 and veg1 endoderm but has cleared from the now ingressed micromere descendants (bottom row Fig. 6B). These results are exactly as expected for the early *blimp1/krox* transcription unit.

Functional characterization of the early form of blimp1/krox

The initial predictions for inputs of *blimp1/krox* within the endomesodermal gene regulatory network relied on perturbation data from experiments using a fusion construct, in which the *Drosophila* Engrailed repressor domain was joined to the DNA binding domain of *blimp1/krox* (Bl1/K-En). In embryos injected with Bl1/K-En mRNA, all direct targets of *blimp1/krox*

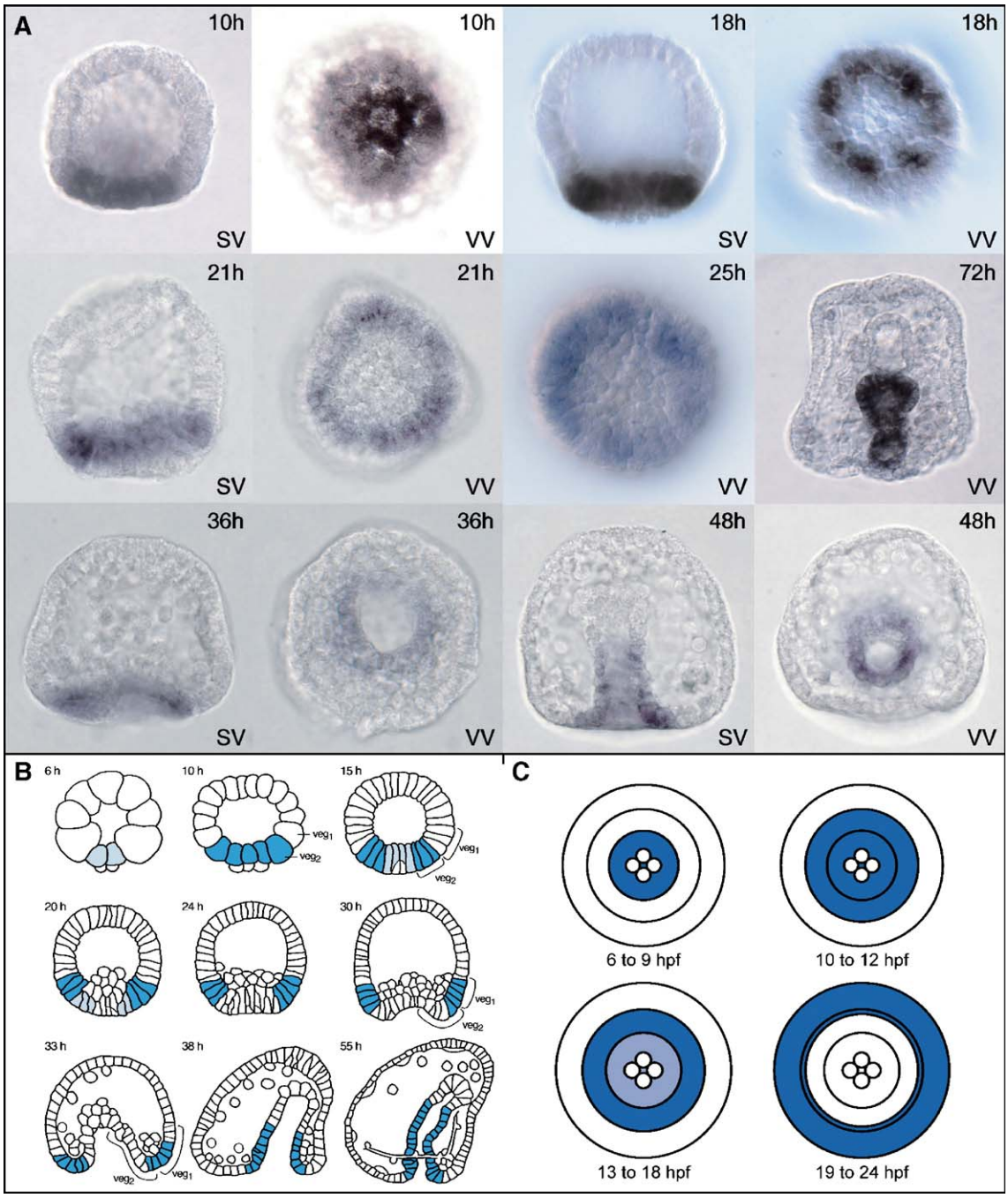


Fig. 5. Spatial expression of *blimp1/krox*. (A) Spatial distribution of *blimp1/krox* transcripts detected by whole mount in situ hybridization (WMISH) using digoxigenin-labeled antisense probe; images of WMISH embryos mounted in 50% glycerol are shown. At 10 hpf, the gene is expressed in the large micromere descendents; at 18 hpf, it is expressed in the veg2 tier of cells, but is no longer observed in the large micromeres; at 21 hpf, it is expressed in the veg1 tier of cells, and expression has faded from the veg2 mesoderm. A similar pattern is seen at 25 hpf. At 36 hpf the gene is expressed in the cells surrounding the blastopore; at 48 hpf, the gene is expressed in the hind and mid gut; and at 72 hpf it is expressed in the mid and hind-gut of the pluteus larva. Bottom right hand corner indicates the view of the embryo displayed (SV, side view; VV, vegetal view). (B) Side view diagram of *blimp1/krox* spatial expression during embryogenesis, based on (A) and on many additional images not shown. (C) Vegetal view diagram of *blimp1/krox* spatial expression.

should be strongly down-regulated with respect to controls, since the Engrailed domain acts as a dominant repressor. Indeed, most genes affected displayed strong down-regulation in these experiments, though in rare cases, an up-regulation occurred, necessarily an indirect effect. In order to determine the real polarity of the endogenous *blimp1/krox* effects on these genes, we studied them again, using MASOs targeted against the *blimp1/krox1b* early gene product, or in combination with *blimp1/krox1a* MASO.

The two alternatively transcribed isoforms utilize different translation initiation sites. Different MASOs can therefore be used in order to block the translation of either message, so that the function of the early and late forms can be established separately. The antisense *blimp1/krox1a* MASO has no

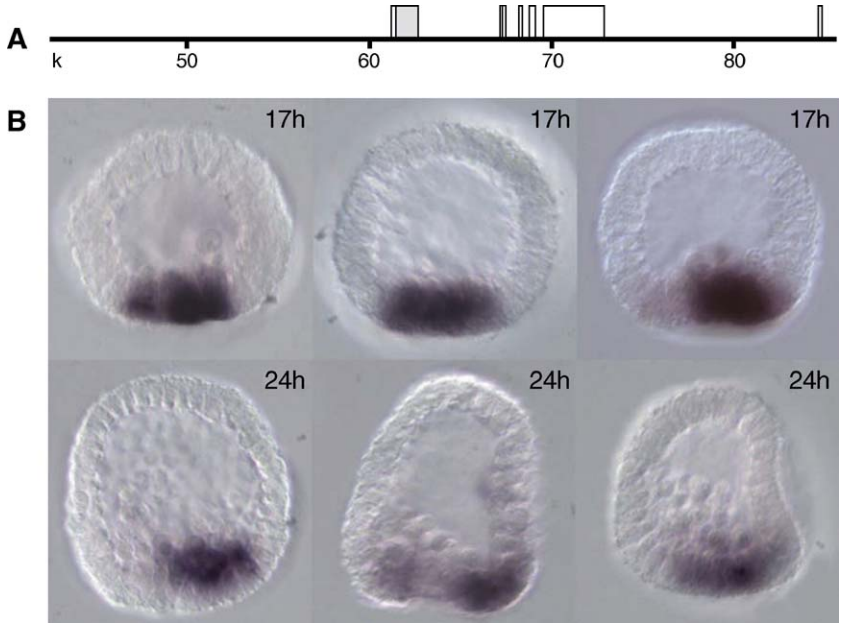


Fig. 6. Expression of *blimp1/krox1b*-GFP knockin BAC in transgenic embryos. (A) Diagram of *blimp1/krox1b*-GFP knockin BAC. The GFP coding sequence was inserted by homologous recombination in place of the coding region for exon 1b. The 5′ UTR was maintained and only a few nonconserved nucleotides from the 3′ end of the exon were removed in the recombination process. (B) GFP expression patterns generated by the *blimp1/krox1b*-GFP knockin BAC are shown, visualized by WMISH using antisense GFP probe. Embryos were 17 and 24 hpf as indicated, and are all shown in side view. Bottom right hand corner indicates the view displayed of the embryo. At 17 hpf, GFP mRNA is present in the large micromeres, but it is absent from their descendants in the 24 hpf embryos, as can clearly be seen once these cells have ingressed. DNA constructs injected into sea urchin zygotes are incorporated in a mosaic fashion (Hough-Evans et al., 1988), thus the transgene expression observed in an individual embryo is a fraction of the complete pattern assembled from observing many embryos.

noticeable phenotype until the gastrula stage. Thereafter, the treated embryos produce pencil-like guts that fail to form the normal tripartite structure (data not shown). This phenotype correlates well with the temporal expression of the gene, since *blimp1/krox1a* mRNA accumulates only after 36 hpf. A similar phenotype is displayed in embryos bearing *brn1/2/4* MASO; as noted above we believe Brn1/2/4 could be a driver of the *blimp1/krox1a* transcription unit (Yuh et al., 2005).

The *blimp1/krox1b* MASO, by contrast, has a strong early phenotype, including a decrease in the thickening of the vegetal plate, and subsequently, lack of a clear veg1 descendant tier of cells in the blastula stage. This is reminiscent of the phenotype of embryos expressing mRNA encoding *blimp1/krox*-En fusions (Davidson et al., 2002a,b), but it is not as strong. At 40 hpf, it is easy to distinguish controls from anti-*blimp1/krox1b* MASO-injected embryos. As can be seen in Figs. 7C, D, anti-*blimp1/krox1b* MASO-injected embryos may display less and more severe phenotypes (compare controls, Figs. 7A, B). In about 50% of cases, a small invagination does appear as in Fig. 7C, although it is much delayed when compared to controls, and it does not ever extend to form a gut. In the remaining 50% of MASO embryos, no invagination occurs at all, and in some cases, cells in the embryos begin to exogastrulate instead (Fig. 7D).

Treatment of embryos with anti-*blimp1/krox1b* MASO affects expression of this gene itself in a striking way. The controls in Figs. 8A–C show normal WMISH patterns of expression observed with *blimp1/krox* probes (cf. Fig. 5). The remaining five panels of Fig. 8 display embryos injected with a MASO targeted to the *blimp1/krox1b* message. These embryos lack the torus of endoderm expression formed in normal embryos by the clearance after 18 hpf of transcripts from the central veg2 mesoderm domain. The inner boundaries of this

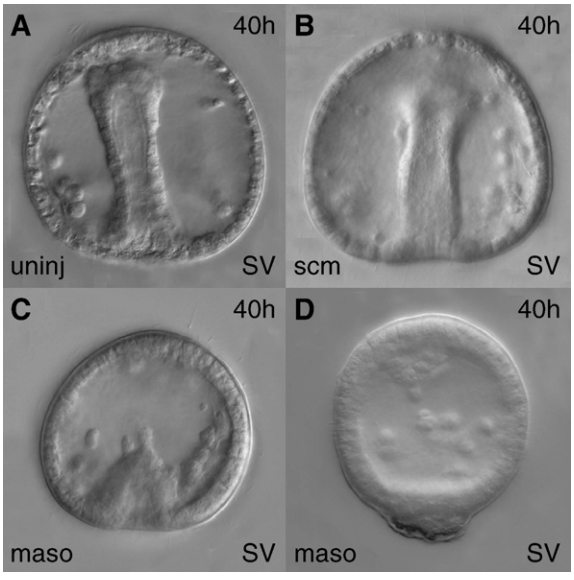


Fig. 7. Phenotypes of embryos bearing *blimp1/krox1b* MASO. Embryos were injected with 2 to 4 pl of a 100-μM stock of the MASO (see Materials and methods for details). (A) Uninjected (uninj), (B) standard control morpholino (scm), and (C, D) *blimp1/krox* MASO embryos. A well-developed gut can be observed in controls (panels A and B). The embryo shown in panel C has formed an invagination, but gut elongation did not proceed beyond formation of a short, truncated archenteron. The embryo shown in panel D formed no invagination, and instead the vegetal most cells are exogastrulating.

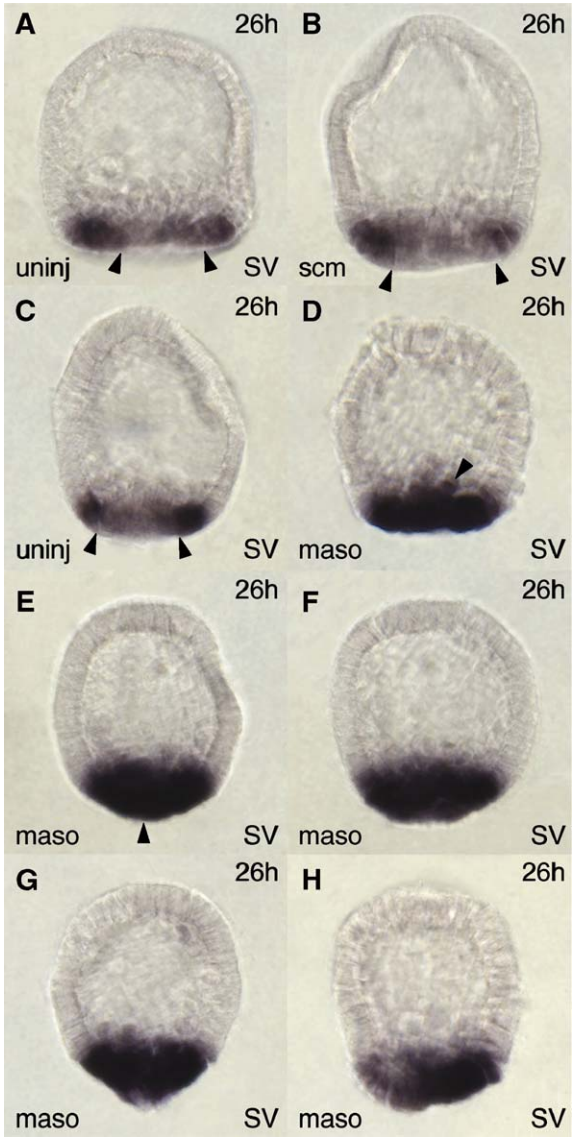


Fig. 8. Negative *blimp1/krox* spatial autoregulation in veg2 mesodermal cells. Embryos were injected with 2 to 4 pl of a 100 μM of anti-*blimp1/krox1b* MASO and 200 μM of anti-*blimp1/krox1a* MASO, and WMISH performed using *blimp1/krox* probes. (A, B) uninjected embryos (uninj); (C) standard control morpholino (scm); and (D–H) *blimp1/krox* MASO. In (A–C), arrows point to boundaries of mesodermal region cleared of signal. In the MASO-treated embryos staining is expanded to the center of the vegetal plate (arrow in E), displaying the ectopic expression of *blimp1/krox* message in the mesodermal veg2 descendents. An ingressed skeletogenic mesenchyme cell expressing *blimp1/krox* is indicated by the arrow in (D).

taurus, i.e., the mesoderm/endoderm interface, are indicated in the controls of Figs. 8A–C, by arrows. In the MASO-treated embryos, *blimp1/krox* expression never clears from the mesoderm, and at 26 hpf intense expression is continuing across the whole of the vegetal plate. The arrow in Fig. 8E, for example, points to the center of the mesodermal domain in a MASO-treated embryo displaying heavy *blimp1/krox* expression in this region. Though we did not explicitly address the requirement of *blimp1/krox* expression for the initial clearance of transcripts from the skeletogenic micromeres, it may operate by a similar mechanism. Thus, for instance, Fig. 8D shows a MASO treated embryo in which ingressed micromeres can be seen expressing *blimp1/krox* ectopically (arrow), which is never normally observed. The subsequent expression of *blimp1/krox* message in the endodermal portion of the veg1 tier also appears to be missing in MASO treated embryos (not shown). In summary, *blimp1/krox* is certainly required to repress its own expression in mesodermal cells of the veg 2 tier. It may also be required for its earlier down-regulation in the large micromeres, and, directly or indirectly, for the activation of its own expression in the endodermal cells of the veg 1 tier. Note that *blimp1/krox* does not repress its own expression within cells of the veg1 or veg2 endodermal territories. The difference in the response of the gene to the *blimp1/krox* factor in the mesoderm, where it acts as a repressor, and in the endoderm, where it does not, must be due to the presence of different co-regulators in these domains.

Computational binding site searches and gel shift analyses indicate that the negative autoregulation of the *blimp1/krox* gene is likely to be direct. There are several sites in the intergenic region surrounding the *blimp1/krox* exons which correspond to the consensus target site sequence for *blimp1/krox* factors, G(A/G)AA(G/C)(G/T)GAAA (Gupta et al., 2001). We found that these sites are bound by a factor the mobility of which is very similar to that binding the *blimp1/krox* sites in a known *otx cis*-regulatory module (Yuh et al., 2004); and that the *blimp1/krox* sites of the *blimp1/krox* gene and those from the *otx* gene regulatory module compete reciprocally (details are given in Figs. 4 and 5 of Supplemental Materials).

Discussion

Alternative regulation of the blimp1/krox splice isoforms

The early and late transcripts of the *blimp1/krox* gene have different lead exons, positioned at widely different locations in the genome (Fig. 1), and are transcribed at different stages in development (Fig. 4). The proteins derived from the two isoforms could be functionally distinct, since their N-terminal sequence differs by the exon 1b-specific peptide (Fig. 2). However, the highly conserved domains, the SET domain, and the DNA binding zinc fingers, are present in both *blimp1/krox1a* and *blimp1/krox1b* proteins. As will be described elsewhere, transcription of the 1a and 1b forms is controlled by entirely distinct regulatory modules, which respond to distinct inputs. Whatever the significance of the N-terminal peptide, if any, the alternative regulatory systems enable the gene to be deployed in very distinct circumstances: early on it functions as one of the β-catenin/Tcf1 cohort of regulatory genes, and as such *blimp1/krox* is among the primary regulators of the zygotic gene regulatory network. The general role of these very early regulators is interpretation of cytoplasmic spatial cues at the beginning of development, here vegetal nuclearization of β-catenin, and installation of the zygotic transcriptional control system. In contrast, the late or 1a form is expressed only in the definitive endoderm of the hindgut and midgut. According to perturbation data from Yuh et al. (2005), the regulatory system controlling 1a expression responds to a Brn1/2/4 input, which is

a midgut and hindgut, and later a midgut specific regulator. Brn1/2/4 controls expression of *endo16*, a downstream differentiation gene, and *blimp1/krox1a* might likewise be used to operate differentiation genes in this phase of its function. The phenotype of the embryos treated with MASO targeted to the late form indicates that expression of this form is required for gut regionalization.

Downstream targets of blimp1/krox expression

As demonstrated in Figs. 5 and 6, *blimp1/krox* message is first expressed in the skeletogenic micromere lineage, then in the secondary mesenchyme mesodermal domain, the cells of which are of independent origin (fourth cleavage micromere vs. macromere), and subsequently in the veg2 endoderm, veg1 endoderm, and gut. The expression domains of the early and late transcripts together account for the overall pattern summarized in Fig. 5C. This gene plays a series of roles in endomesoderm specification. Its initial function in the skeletogenic and then the veg2 endomesodermal cells is to drive expression of the *wnt8* gene, into which it has a direct *cis*-regulatory input (Minokawa et al., 2005). Thus, *blimp1/krox* contributes to the essential intercellular Wnt8-βcatenin feedback loop required for expression of important regulatory genes in all of the domains of the endomesoderm (Davidson et al., 2002a,b; Oliveri and Davidson, 2004). Later, the *blimp1/krox* gene generates an essential input into the regulatory apparatus that governs endoderm specification. Here, it operates upstream of an important, highly conserved network subcircuit composed of *otx*, *gatae*, *brachury* and *foxa* genes that drives endoderm specification, as discovered earlier (Davidson et al., 2002a,b; Hinman et al., 2003). Of these genes, it provides a direct input into the *cis*-regulatory module controlling endodermal expression of the β*1/2otx* transcript. In addition, in the veg2 and veg1 endoderm, *blimp1/krox* is apparently a transcriptional activator of *eve* and of *hox11/13b*. From the mesenchyme blastula stage on, expression of both of these genes ceases in the veg2 endoderm, and appears instead in the veg1 endoderm. These are all functions mediated by the early or 1b form of *blimp1/krox* protein. With respect to the gut-specific late form, only the Brn1/2/4 input into the *cis*-regulatory module controlling its expression is so far established, and none of its downstream targets are yet known.

The network linkages of *blimp1/krox* can now be summarized as in Fig. 9, which includes genes known or suspected to be either immediately upstream or downstream of this gene. This diagram is in the form of a view from the genome (Bolouri and Davidson, 2002; Longabaugh et al., 2005), such that all regulatory linkages between genes are seen at once irrespective of the time or subdomain where they are expressed. For views that specify the interactions occurring in any particular spatial and temporal domain, and which include all genes in the network, the reader may consult the interactive model on the gene regulatory network website (http://sugp.caltech.edu/endomes/).

The blimp1/krox negative autoregulatory loop

We found earlier (Davidson et al., 2002a) that the *blimp1/krox* gene is strongly repressed by an Engrailed-*blimp1/krox* fusion, an indication that a *cis*-regulatory module of this gene might include autoregulatory target sites for its own product. The MASO experiments reported here (Fig. 8) decisively demonstrate that this autoregulation is negative: *blimp1/krox* represses itself, directly or indirectly. Very possibly, the interaction is a direct one, though the demonstration that this

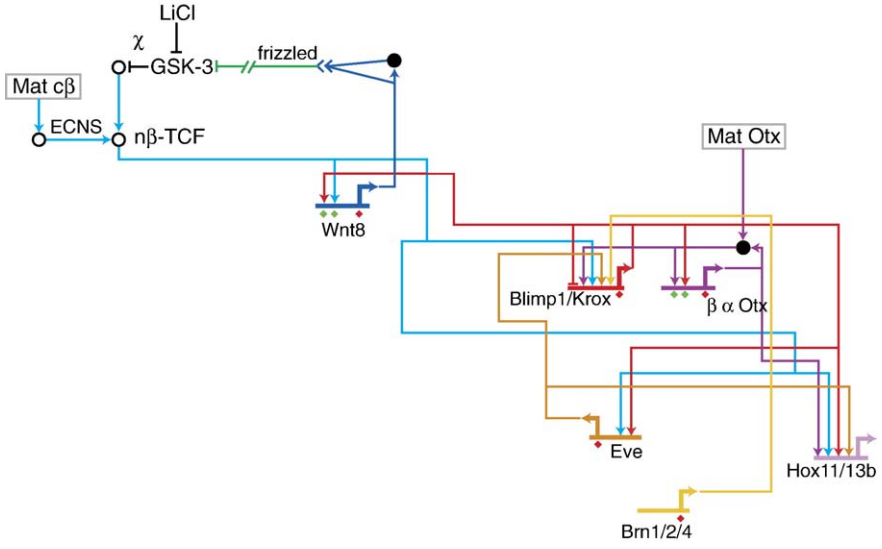


Fig. 9. Linkages of the *blimp1/krox* gene in the endomesoderm gene regulatory network. A view from the genome network diagram (Bolouri and Davidson, 2002; Longabaugh et al., 2005) highlights the upstream and downstream connections of *blimp1/krox*. This view shows all functional connections, independent of when and where these interactions are taking place. There are five unique known inputs into *blimp1/krox* (including both the 1a and 1b regulatory systems). Outputs from the *blimp1/krox* gene are shown in red. There are three known downstream target genes encoding transcription factors (aside from itself) which are likely to be directly regulated by *blimp1/krox*, i.e., *otx*, *hox11/13b*, and *eve*, plus the *wnt8* gene. Note the negative autoregulatory loop of *blimp1/krox* by which it represses its own transcription. Other important features displayed are the cross-regulatory loops formed with *otx* and *eve*.

is the case awaits the *cis*-regulatory identification of the responsible target sites. Thus, there are nearby potential target sites which match the canonical sequence recognized by Blimp1 proteins, and competition gel shift experiments show that these indeed specifically interact with the sea urchin *blimp1/krox* factor (Supplemental Materials, Figs. 4 and 5). Direct or not, the negative autoregulation of *blimp1/krox* is evidently the cause of the dynamic clearance of its transcripts from the veg2 mesodermal domain in the late blastula stage, since elimination of expression at the protein level by MASO treatment blocks this clearance (Fig. 8). As discussed in text, it is likely that the same mechanism is responsible for the earlier clearance of *blimp1/krox* transcripts from the skeletogenic micromere lineage as well. In spatial terms, the expression of this gene describes a wave-like form, in that it is activated in the micromere lineage between 6 and 9 hpf, but the transcripts have disappeared from this lineage sometime prior to 18 h; similarly, it is activated in the veg2 mesodermal lineage by about 10 hpf, and the transcripts have disappeared from this domain by 21 hpf, as discussed above. Were the mRNA to vanish the moment the gene is turned off in consequence of its own transcription, the periodicity of this wave would be expected to be about 2 to 3 h rather than at least 9 h (Bolouri and Davidson, 2003). The difference might indicate that the negative autoregulation is in fact indirect, but the most likely explanation is that the mRNA has a several-hour half life. If this were true then in each domain the gene might be expressed only for a short burst of a few hours. This is the phase of its activity when *blimp1/krox* drives the *wnt8* self-reinforcing loop (Minokawa et al., 2005). The *wnt8* gene similarly begins operation in the skeletogenic micromeres, expands to the veg2 mesoderm, turns off in the micromeres, and later turns off in the veg2 mesoderm while expanding into the veg2 endoderm and then veg1 endoderm. Another *blimp1/krox* target gene, *eve*, describes a very similar dynamic pattern of expression (Ransick et al., 2002). Thus, the negative autoregulation of *blimp1/krox* could provide part of the causal explanation of this progressive spatial expression pattern, that is, it could explain the progressive, concentric clearance of transcripts that all three genes display.

In summary, this work illustrates the multiplicity of functions that a single regulatory gene may execute over the course of a couple of days of embryonic development. The *blimp1/krox* gene is alternatively transcribed, under diverse regulatory controls, at different stages and in different places. After gastrulation, the late form participates in gut regionalization. The early transcript form has diverse roles: initially it provides a spatially and temporally dynamic input into the Wnt8–Tcf1 regulatory loop, which literally defines the endomesoderm; and then a few hours later, it operates to drive an endoderm specification network subcircuit.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2006.02.021.

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