In addition to confirming theoretical genetic models for speciation by hybrid recombination, this study demonstrates the utility of comparative linkage mapping for studying the genomic processes accompanying or facilitating different modes of speciation. Not only can these maps be used to infer precisely the genomic structural changes accompanying speciation, but they also allow the genomic contribution of parental taxa to hybrid species or introgressant populations to be determined on a chromosome by chromosome basis, as well as within chromosomes. Future comparative mapping studies of species-level questions could be enhanced by the development of microsatellite loci[20], because most of these loci are likely to be polymorphic even in very narrow intraspecific mapping populations, and homology among loci can be assumed without additional experiments such as those required here (Fig. 1 and Supplementary Information). Microsatellite markers are currently being developed for *Helianthus* mapping populations. □

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Defective haematopoiesis in fetal liver resulting from inactivation of the EKLF gene

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ERYTHROID Krüppel-like factor (EKLF) was originally isolated from erythroid cell RNA by differential screening and shown to be erythroid-specific, although a low level of EKLF was found in mast cell lines[1,2]. EKLF contains three zinc-fingers homologous to those found in the Krüppel family of transcription factors. Because it binds the sequence CCACACCCT, EKLF may affect erythroid development as a result of its ability to bind to the CAC box in the promoter of the β-globin gene[1,2]. Mutation of this element leads to reduced β-globin expression[3-5] and it appears to mediate the effect of the globin locus control region on the promoter[6]. Here we inactivate the EKLF gene through insertion of a *lacZ* reporter gene by homologous recombination in embryonic stem (ES) cells. Heterozygous EKLF^{+/−} mice show that the reporter gene is expressed in a developmentally specific manner in all types of erythroblasts in the fetal liver and adult bone marrow. Homozygous EKLF^{−/−} mice appear normal during the embryonic stage of haematopoiesis in the yolk sac, but develop a fatal anaemia during early fetal life when haematopoiesis has switched to the fetal liver. Enucleated erythrocytes are formed but these do not contain the proper amount of haemoglobin. We conclude that the transcription factor EKLF is essential for the final steps of definitive erythropoiesis in fetal liver.

Using the known EKLF sequence[1], we isolated a cosmid from a 129 mouse DNA library and inserted a *lacZ* reporter gene[7] in frame and a marker gene to select for neomycin resistance[8] at the ATG start codon (Fig. 1). Transfection of the linearized plasmid into E14 embryonic stem cells[9] and double selection (neo[+], tk[−])[10] resulted in 60% homologous recombinants, as determined by Southern blot analysis of individual clones (Fig. 1). Six of the ES clones with a 70–90% normal karyotype were injected into mouse blastocysts[11], and three (D1, D8 and D32) resulted in chimaeric mice that transmitted the EKLF[−] allele through the germ line (Fig. 1). The three founder lines were all bred to different background mice (FVB, BL6 and CBA). All of these combinations resulted in the same phenotype and therefore the different mice will not be discussed separately.

Analysis of the different developmental stages of the mice showed that the *lacZ* coding unit driven by the EKLF gene was first expressed in all types of erythroblasts (not shown) in the fetal liver (Fig. 2a) through to adult bone marrow. The gene was not expressed in the embryonic yolk sac or blood (as shown by the lack of LacZ staining, which does not exclude a low level of EKLF expression; Fig. 2b) or in any other tissue during
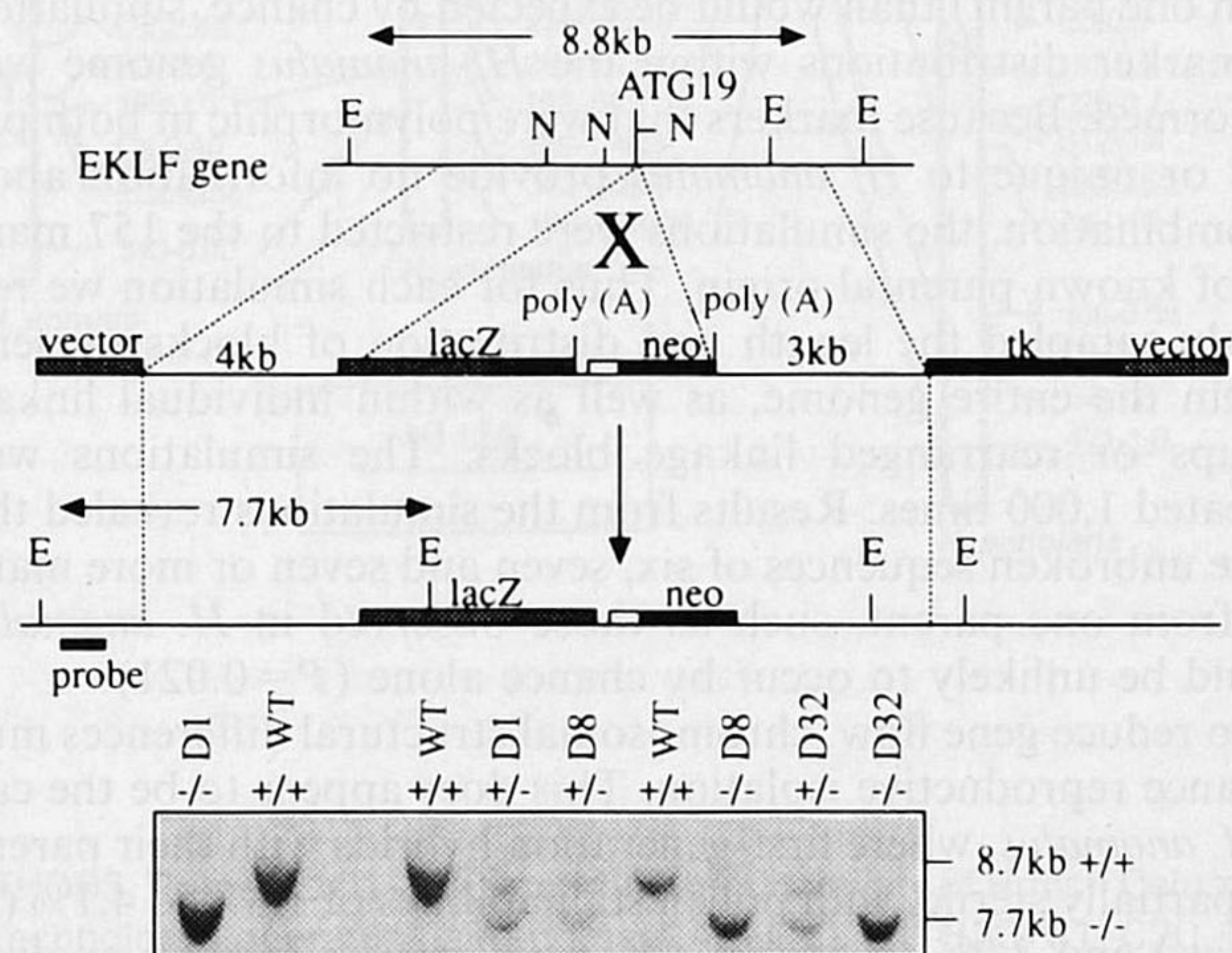

FIG. 1 Homologous recombination into the murine EKLF gene. The coding region of the bacterial *lacZ* gene[7] and the neo-selectable marker[10] were isolated as a 4.6-kb (NcoI compatible) BspHI fragment (D.M., unpublished work) and cloned into a partially digested 7-kb NcoI fragment at methionine 19 of the EKLF gene[1]. The linearized EKLF/*lacZ*/neo plasmid, which already contained the herpes simplux virus thymidine kinase (tk) gene, was introduced into E14 ES cells[9]. Homologous recombinants were detected by hybridization with internal (*lacZ*) and external flanking probes. After creating chimaeric mice by ES cell injection into blastocysts[11], three separate founder lines were established. Bottom panel, Southern blot hybridization with a 5' flanking probe of the three founder lines. The wild-type (WT) EKLF allele is detected as an 8.7-kb fragment; the recombined allele is detected as a 7.7-kb fragment, because of the introduction of a novel EcoRV site. E, EcoRV; N, NcoI.
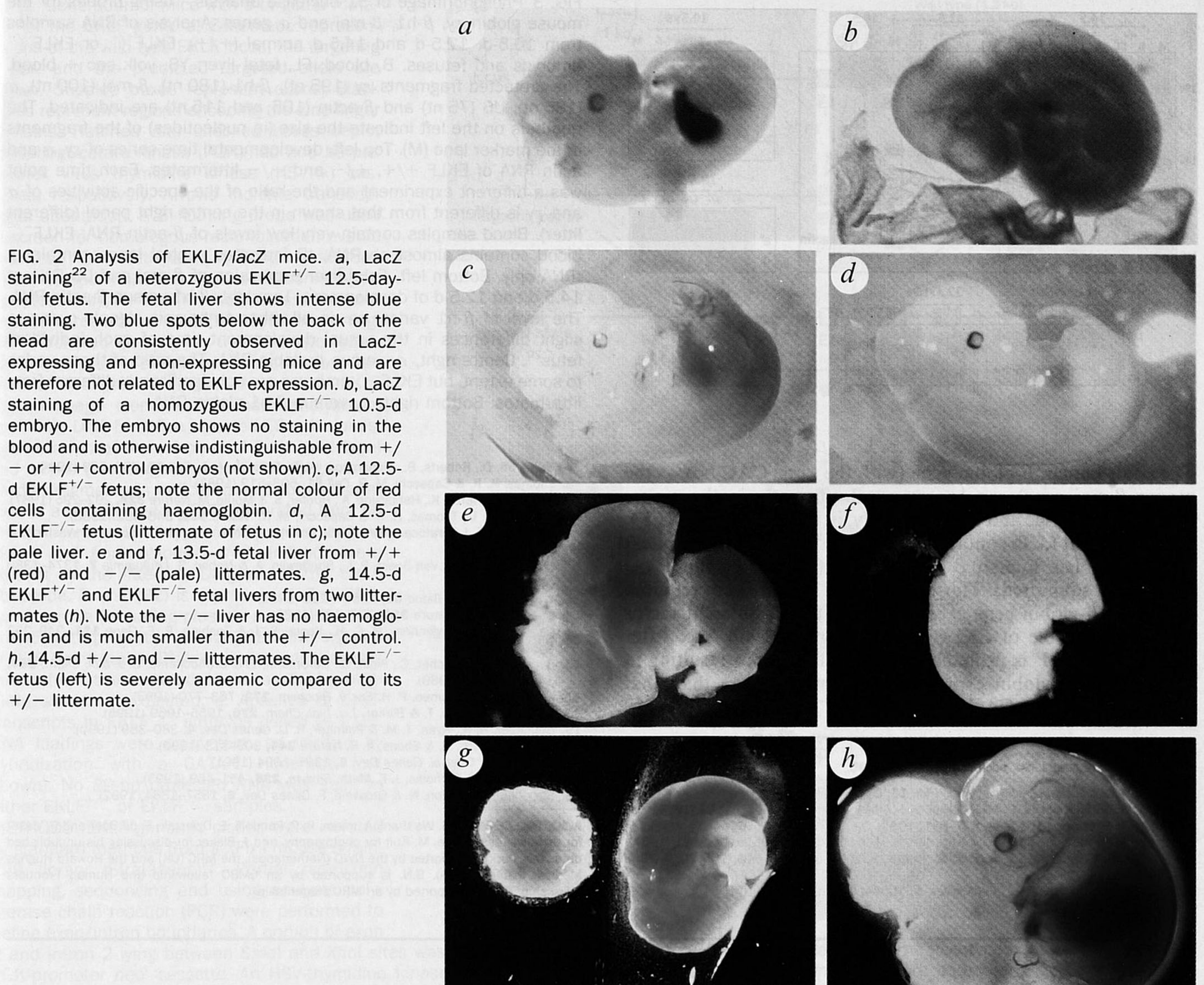
FIG. 2 Analysis of EKLF/lacZ mice. a, LacZ staining[22] of a heterozygous EKLF$^{+/-}$ 12.5-day-old fetus. The fetal liver shows intense blue staining. Two blue spots below the back of the head are consistently observed in LacZ-expressing and non-expressing mice and are therefore not related to EKLF expression. b, LacZ staining of a homozygous EKLF$^{-/-}$ 10.5-d embryo. The embryo shows no staining in the blood and is otherwise indistinguishable from +/− or +/+ control embryos (not shown). c, A 12.5-d EKLF$^{+/-}$ fetus; note the normal colour of red cells containing haemoglobin. d, A 12.5-d EKLF$^{-/-}$ fetus (littermate of fetus in c); note the pale liver. e and f, 13.5-d fetal liver from +/+ (red) and −/− (pale) littermates. g, 14.5-d EKLF$^{+/-}$ and EKLF$^{-/-}$ fetal livers from two littermates (h). Note the −/− liver has no haemoglobin and is much smaller than the +/− control. h, 14.5-d +/− and −/− littermates. The EKLF$^{-/-}$ fetus (left) is severely anaemic compared to its +/− littermate.

development. The heterozygous mice appeared to be healthy, they were fertile and their haematocrit, mean corpuscular volume and absolute and differential blood cell counts were normal (not shown). Cell numbers in spleen and bone marrow were the same as in wild-type littermates.

Crossing of heterozygous EKLF$^{+/-}$ animals did not result in live-born homozygous EKLF$^{-/-}$ mice. At earlier stages, embryos at day 10.5 appeared normal (Fig. 2b), containing apparently normal nucleated embryonic (E10.5) erythrocytes in the yolk sac and blood (data not shown). But after switching of embryonic haematopoiesis to the fetal liver, the EKLF deletion gives rise to a severe phenotypic effect (Fig. 2c, d, h). The fetal liver contains normal numbers of the various erythroblasts, including enucleated cells that lack their full complement of haemoglobin, resulting in a rapid anaemia, erythroblastosis and growth retardation. The embryo dies around day 14, presumably because the remaining circulating blood cells can no longer sustain life (see Fig. 2g, h for example). Although the growth and development of the fetal liver is severely affected (Fig. 2e–g), a normal number of colony-forming cells were seen in the early fetal liver, indicating that the progenitor cell compartment[12] is not affected (about 3,000 CFU-C per fetal liver in EKLF$^{-/-}$ and EKLF$^{+/+}$ mice; data not shown). To confirm that the primitive (embryonic) blood cells, as opposed to the definitive (fetal liver) cells, are normal, we analysed the different β-globin-like genes using U6 or β-actin genes as a control. S1 nuclease protection

experiments (Fig. 3) show the embryonic εy and β-h1 RNAs to be present, with the expected persistence of the signals in the circulating embryonic blood in the fetus[13,14]. However, only small amounts of mature β-maj RNA (<10-fold) can be detected in the fetal liver of EKLF$^{-/-}$ mice, in agreement with the phenotype already described. The α-globin gene is clearly expressed in the embryonic yolk sac, fetal liver and blood of all mice. We have also probed for the expression of other erythroid genes (CA1 and PBGD) containing a CAC box with a single base EKLF consensus mismatch[15-17], and both of these are expressed at high levels in the fetal liver, although the PBGD gene expression may be slightly reduced (data not shown).

We conclude that EKLF is essential for the last steps of differentiation of mature erythroid progenitors and that it is unlikely to be important in the early events that lead to red cell differentiation. It is not yet known which genes are directly regulated by EKLF, but its presence in definitive erythropoiesis (fetal liver and bone marrow) and absence (or low level[18]) in primitive erythropoiesis (embryonic yolk sac) and its expression pattern in the differentiation of red cells, all suggest that it targets the genes involved in haemoglobin production. Our results combined with those from transfection experiments[1,2] indicate that EKLF could be involved in the expression of adult β-globin genes by specifically recognizing the CAC box in the fetal/adult stages. This CAC box is slightly different from those in the embryonic globin genes and from that in the α-globin genes.
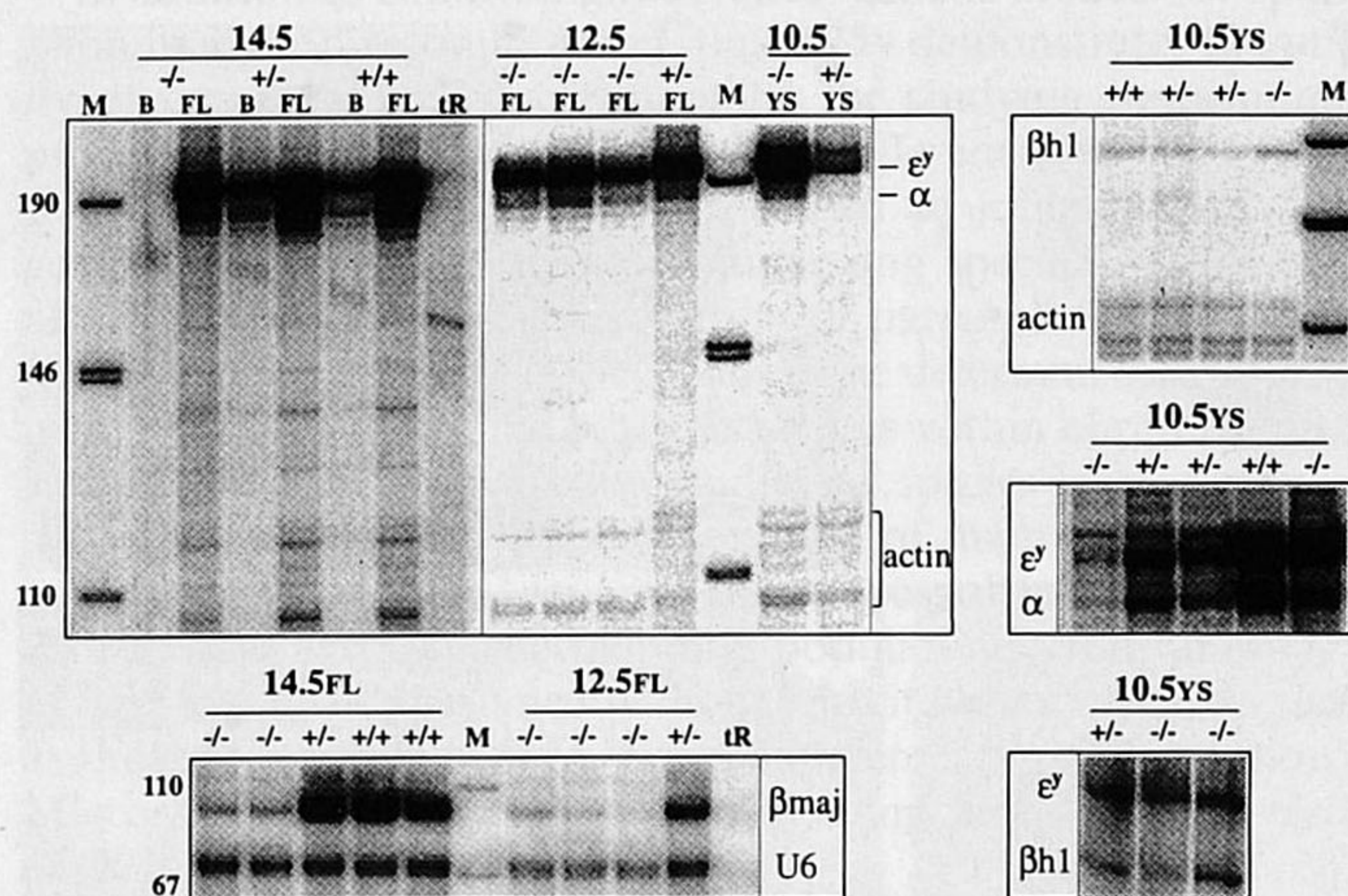
FIG. 3 Phosphorimage of S1 nuclease analysis[23] using probes for the mouse globin εy, β-h1, β-maj and α genes. Analysis of RNA samples from 10.5-d, 12.5-d and 14.5-d normal (+/+), EKLF^{+/-} or EKLF^{-/-} embryos and fetuses. B, blood; FL, fetal liver; YS, yolk sac + blood. The protected fragments εy (195 nt), β-h1 (180 nt), β-maj (100 nt), α (185 nt), U6 (75 nt) and β-actin (105 and 115 nt) are indicated. The numbers on the left indicate the size (in nucleotides) of the fragments in the marker lane (M). Top left, developmental time series of εy, α and actin RNA of EKLF +/+, +/− and −/− littermates. Each time point was a different experiment and the ratio of the specific activities of α and εy is different from that shown in the centre right panel (different litter). Blood samples contain very low levels of β-actin RNA; EKLF^{-/-} blood contains almost no RNA. tR indicates control lanes containing tRNA only. Bottom left, Fetal liver expression of β-maj and U6 RNA at 14.5 d and 12.5 d of development. Top right, β-h1 versus β-actin RNA. The level of β-h1 varies, as do all other embryonic signals owing to slight differences in the actual development time of each individual fetus[20]. Centre right, εy versus α-globin RNA. The ratio of these varies to some extent, but EKLF^{-/-} embryos are not significantly different from littermates. Bottom right, εy versus β-h1 globin RNA.

The adult human β-globin gene (with the same CAC box as the mouse β-maj gene) can be expressed efficiently in the mouse embryonic yolk sac and blood (namely, in tissue lacking EKLF), both as a small LCR-containing construct[19-21] and in the context of the complete human β-globin locus (N. Dillon and F.G., manuscript submitted). This suggests that EKLF is not required *per se* for β-globin gene expression and that a different factor may fulfil the role of EKLF in the embryonic yolk sac. After that stage, EKLF is probably essential, its absence resulting in a reduction of globin expression even more severe than that caused by β-globin CAC-box mutations[3-5]. ☐

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Lethal β-thalassaemia in mice lacking the erythroid CACCC-transcription factor EKLF

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GLOBIN genes are regulated in a tissue-specific and developmental stage-specific manner, with the β-globin gene being the last to be activated in the β-gene cluster[1]. CACCC-nucleotide sequences, which bind multiple nuclear proteins, including ubiquitously expressed Sp1 and erythroid Krüppel-like factor (EKLF), are among the *cis*-regulatory sequences critical for transcription of globin and non-globin erythroid-expressed genes[2-5]. To determine the function of EKLF *in vivo*, we created mice deficient in EKLF by gene targeting[6]. These embryos die of anaemia during fetal liver erythropoiesis and show the molecular and haematological

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features of β-globin deficiency, found in β-thalassaemia. Although it is expressed at all stages, EKLF is not required for yolk sac erythropoiesis, erythroid commitment or expression of other potential target genes. Its stage-specific and β-globin-gene-specific requirement suggests that EKLF may facilitate completion of the fetal-to-adult (haemoglobin γ to β) switch in humans.

Using homologous recombination in embryonic stem (ES) cells, we inactivated the mouse EKLF gene by replacing a portion of the zinc-finger DNA-binding domain with a neomycin-resistance *neo*R cassette (Fig. 1a). ES cells with a targeted allele (Fig. 1b) were used to generate heterozygous (EKLF^{+/-}) mice which were bred together to produce homozygous (EKLF^{-/-}) mice (Fig. 1c). Stable EKLF transcripts were not detected in EKLF^{-/-} embryos (Fig. 1d, e).

EKLF^{-/-} embryos appeared to be normal during the yolk sac stage, but became progressively more anaemic from embryonic-day 11 (E11) to E15, paralleling the dependence on the switch from embryonic to fetal-liver-derived erythropoiesis (Fig. 2a–e; Table 1). Heterozygous mice were unaffected. EKLF^{-/-} embryos died by E16. Peripheral blood of E15 EKLF^{-/-} embryos revealed residual embryonic red cells of normal appearance, but abnormally small and irregularly shaped red cells of fetal liver origin (Fig. 2d–e). Increased numbers of nucleated fetal liver erythroblasts were also present in the circulation. Fetal livers of EKLF^{-/-} embryos were of normal size and replete with erythroid precursors (see below and Table 1), but erythroid maturation, notably haemoglobin accumulation, was defective