mV) reduction in the afterhyperpolarization (AHP). Using a low concentration of TEA (1 mM), which preferentially blocks the Ca^{2+}-dependent K^+ current in *Aplysia* sensory neurons, we could mimic the change in the AHP without any observable effect on transmitter release ($n = 6$).
31. In 10 μM nifedipine, the average amplitude of the PSP was 89.1 ± 9.0%, not significantly different from the average control value ($t = 1.21$; $n = 5$). When pooled with the data obtained from experiments with 5 μM nifedipine, the results were still not significant (see text).
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33. In the presence of 10 μM 5-HT, application of 10 μM nifedipine did not significantly alter the average amplitude of the synaptic potential as percent of the average control value in 5-HT (98.0 ± 3.2%; $t = 0.63$; $n = 4$). Moreover, when pooled with the data from experiments with 5 μM nifedipine, the result was still not significant (see text).
34. Because FMRFamide, an agent that causes presynaptic inhibition, reduces Ca^{2+} current, we might suspect that 5-HT, which causes presynaptic facilitation, would enhance this current. Although modulation of Ca^{2+} current by 5-HT was not detected previously (*42*), the isolation of the Ca^{2+} current with the whole-cell recording technique (*14*) allowed us to detect a modulation of the Ca^{2+} current by 5-HT. Contrary to expectation, 5-HT does not modulate the rapidly inactivating current. Rather, it selectively enhances a current that is similar and possibly identical to the nifedipine-sensitive, slowly inactivating component of current (B. Edmonds, unpublished data). Despite this enhancement by 5-HT, blockage of this current with nifedipine does not significantly affect the expression of presynaptic facilitation. Therefore, modulation of a given species of Ca^{2+} current does not necessarily imply that modulation of that particular current is important for transmitter release.
35. The sensory neuron was voltage-clamped with a single microelectrode, and release was monitored with an electrode in the follower cell. We unmasked the Ca^{2+} current of the presynaptic cell by perfusing with a TEA-containing, Na^+-free solution (Figs. 1 and 2A), to which we added 100 mM Na^+ to carry the postsynaptic current.
36. Although we did not rigorously test the selectivity of Cd^{2+} as a Ca^{2+} channel-blocker in sensory neurons, it was clear that both of the components that we had identified were at least partially blocked by 10 μm Cd^{2+}. For example, Fig. $4C_1$ shows that the sustained current at the end of the pulse is almost completely blocked by nifedipine, but is only partially reduced by Cd^{2+}. By contrast, both Cd^{2+} and nifedipine inhibited the peak current to the same extent, suggesting that Cd^{2+} also reduced the rapidly inactivating component.
37. These experiments were conducted in the presence of 5-HT to maximize PSP amplitudes, partially counteracting the effect of homosynaptic depression.
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44. We thank S. A. Siegelbaum, J. Koester, and P. Pfaffinger for comments on an earlier draft of this paper; H. Ayers and A. Krawetz for typing the manuscript; and K. Hilten and S. Mack for preparing the figures. Supported by the Howard Hughes Medical Institute.

2 March 1990; accepted 27 June 1990

Autonomous Developmental Control of Human Embryonic Globin Gene Switching in Transgenic Mice

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The mechanisms by which expression of the β-like globin genes are developmentally regulated are under intense investigation. The temporal control of human embryonic (ε) globin expression was analyzed. A 3.7-kilobase (kb) fragment that contained the entire human ε-globin gene was linked to a 2.5-kb cassette of the locus control region (LCR), and the developmental time of expression of this construct was studied in transgenic mice. The human ε-globin transgene was expressed in yolk sac–derived primitive erythroid cells, but not in fetal liver or bone marrow–derived definitive erythroid cells. The absence of ε gene expression in definitive erythroid cells suggests that the developmental regulation of the ε-globin gene depends only on the presence of the LCR and the ε-globin gene itself (that is, an autonomous negative control mechanism). The autonomy of ε-globin gene developmental control distinguishes it from the competitive mechanism of regulation of γ and β-globin genes, and therefore, suggests that at least two distinct mechanisms function in human hemoglobin switching.

IN HUMANS, PRIMITIVE ERYTHROPOIESIS takes place in the blood islands of the embryonic yolk sac. Definitive erythropoiesis originates in the fetal liver and shifts to the bone marrow at around the time of birth. At different stages of ontogeny, human erythroid cells contain different β-globin chains, ε chain synthesis is restricted to embryonic cells, $^{G}\gamma$ and $^{A}\gamma$ chains predominate in the fetal stage, and the δ and β chains are maximally produced in the adult. The corresponding genes are arranged in a single locus in the order that they are expressed during ontogeny: 5′-ε, $^{G}\gamma$, $^{A}\gamma$, δ, β-3′.

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The activation and high-level transcription of the β-globin locus are thought to be dependent on the locus control region (LCR). This segment of DNA, which is located 6 to 20 kb upstream of the ε gene, is characterized by a series of erythroid-specific hypersensitive sites (*1*). Reverse genetics has shown that the LCR confers high-level, position-independent, copy number–dependent expression on a linked β-globin gene in transgenic mice (*2*). The LCR effect appears to be dominant, as it confers high-level, erythroid-specific expression on a number of cis-linked heterologous erythroid (*3*), housekeeping (*4*), and nonerythroid (*5*) genes. Experiments in transgenic mice show that linkage of the LCR to the individual human fetal (γ) or adult (β) globin genes results in their expression at all stages of mouse development (*6*, *7*). Correct developmental regulation is restored in constructs that contain both the γ and β genes. This suggests that the fetal-to-adult globin gene switch is mediated by a reciprocal mechanism in which the genes compete for the influence of the LCR (*7*).

To investigate the nature of the developmental control of the human embryonic globin gene, we linked the human ε-globin gene to the LCR and analyzed its stage-specific expression in transgenic mice. A 3.7-kb Eco RI fragment that contained the entire human ε-globin gene (*8*) was linked to a 2.5-kb cassette (*9*) that contained hypersensitive sites I to IV of the human

Table 1. Human embryonic gene expression in the blood of transgenic mice. Human ε expression could not be quantitated in the liver since it could not be detected in either long exposure or by scintillation counting. The absence of human ε expression in definitive erythroid cells was further confirmed by RNase protection analysis of the blood of adult μLAR transgenic ε mice. In three experiments ε expression could not be detected even when ten times the normal amount of RNA was used in the analyses. The change in expression from 29% at d11 to 0.28% at d14 represents a reduction in human ε expression by 100-fold during development. As shown in Fig. 4, similar reductions are seen in the endogenous mouse embryonic globin genes (ζ, ϵ^{y}, βh1).

Blood sample date	Gene expression (cpm)		Gene expression human ε/ (mouse α + ζ)	
	Human ε	Mouse α + ζ	Percent	Corrected for gene copies
d11	3,605	24,691	14.6	29.2
d14	65	45,727	0.142	0.284

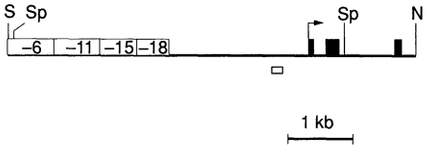

Fig. 1. Map of the μLCR-ε construct used in transgenic experiments. The hypersensitive sites I to IV are shown in open boxes and are designated by their native positions relative to the human ε gene. The ε-globin gene fragment is indicated by a solid line, and its exons are shown as solid boxes. This fragment contains 2 kb of upstream and 0.3 kb of downstream sequences. The hatched box under the diagram indicates the location of the Cla I–Eco RV fragment used as a probe in Southern (DNA) blot analysis. The restriction sites used for the Southern blot analysis and purification of the fragment from the vector are shown. N, Not I; S, Sal I; Sp, Spe I. The LCR was previously known as LAR (locus activation region) or DCR (dominant control region). The new term LCR (locus control region) was agreed on by participants of the 7th Conference on Hemoglobin Switching (8 to 11 September 1990).

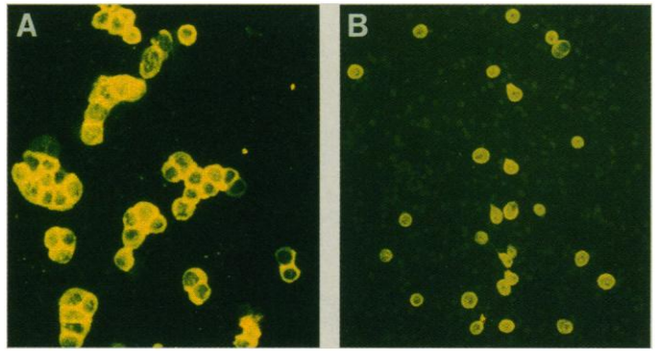

Fig. 2. Immunofluorescence analysis of yolk sac (**A**) and placental (**B**) blood of d9 transgenic embryos with anti-ε. The large nucleated primitive erythroid cells in both preparations stained brightly. The unstained nontransgenic maternal erythrocytes (small enucleate cells) in the placental blood provided a negative control for antibody staining.

β-globin LCR (Fig. 1) (μLCR). This construct (μLCR-ε) (*10*) was microinjected into the male pronucleus of mouse zygotes to produce transgenic embryos. A series of embryos was isolated at day 9 (d9) of gestation. Of the 23 embryos obtained, four were transgenic. Human globin gene expression in these embryos was analyzed by immunofluorescence. A cellular smear of embryonic blood was stained with monoclonal antibody to the human ε-globin chain (anti-ε) (Fig. 2). At d9 of gestation, the blood was composed exclusively of primitive erythrocytes, which contain mouse embryonic globin and derive from the blood islands of the yolk sac (*11*). The vast majority of these primitive cells reacted positively with anti-ε, indicating that the human ε-globin gene linked to the LCR was expressed in primitive erythrocytes (*12*).

We next examined whether μLCR-ε expression was restricted to primitive erythropoiesis or persisted in definitive erythroid cells. At d14 of mouse development, the major site of definitive erythropoiesis is the fetal liver (*11*). The μLCR-ε was reinjected to obtain d14 embryos; 2 of 17 embryos were transgenic. Immunofluorescence analysis was performed with fetal liver–derived cellular smears (Fig. 3). In addition to definitive fetal liver erythroblasts, the smear contained some primitive erythrocytes of yolk sac origin, which are still present in the bloodstream at d14 and circulate through the liver. Whereas these primitive cells stained intensely with anti-ε, the definitive fetal liver erythroid cells did not, indicating that μLCR-ε was expressed exclusively in primitive erythroid cells.

Human ε expression was analyzed at the RNA level in established lines of transgenic mice that contained μLCR-ε. We examined globin expression in staged embryos derived from the breeding of these transgenic mice. A male that contained two intact transgene copies was mated with several females. Females were killed at recorded times after the detection of vaginal plugs, and their embryos were removed. The RNA was extracted from erythroid material (peripheral blood, fetal liver, or both) of the transgenic embryos. Globin gene expression was analyzed by ribonuclease (RNase) protection assays performed with probes that were specific for the human ε-globin as well as endogenous mouse globin transcripts.

Early in ontogeny the blood of the mouse embryo is exclusively populated by primitive erythroid cells that express mouse embryonic globin (ε^y, βh1, ζ). These primitive erythroid cells are gradually replaced by definitive erythroid cells that are produced initially in the fetal liver around d10. The replacement of primitive erythroid cells by definitive erythroid cells was reflected by a decrease in mouse embryonic transcripts (Fig. 4). A similar decrease in human ε-globin mRNA was also seen (Table 1), suggesting that it was regulated as are the embryonic globin genes in mice (*13*). The restriction of human ε-globin gene expression to primitive erythroid cells was confirmed by analysis of RNA from definitive erythroblasts from fetal liver. The presence of definitive erythroid cells in these samples was documented by the successful protection of mouse α-globin mRNA. In contrast, neither human (ε) nor mouse (βh1, ε^y, ζ) embryonic globin transcripts were detected in definitive erythroblasts (Fig. 4).

These results indicate that the ε gene responds to the influence of the LCR in primitive erythroid cells, but escapes the LCR effect in definitive erythroid cells. Because the LCR is active in definitive erythroid cells (*2, 7*), the decrease in expression of the LCR-linked ε gene suggests two things. First, developmental regulation of the ε gene is autonomously controlled; that is, the ε gene contains sufficient information for its own developmental regulation. Second, the developmental regulation of the ε-globin gene is mediated by a negative control mechanism that cannot be overcome by the LCR (*14*).

Our results suggest that there are at least

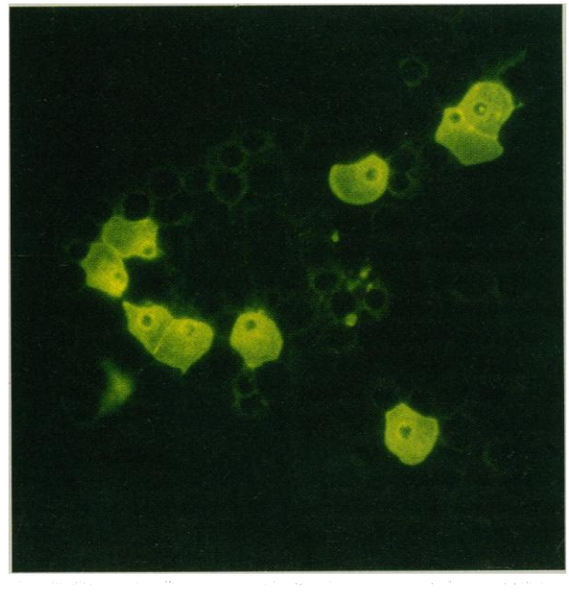

Fig. 3. Immunofluorescence analysis of d14 definitive erythroblasts from fetal liver. The definitive erythroid cells did not stain with the anti-ε. The brightly stained primitive erythroid cells that contaminated the liver provided a positive control for antibody staining.

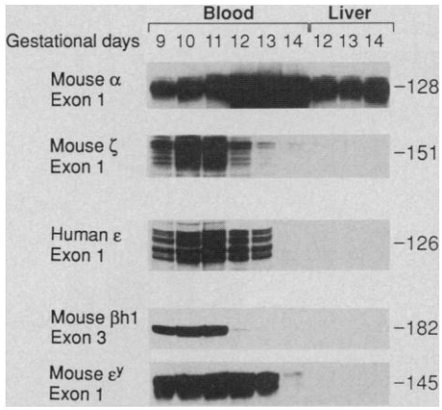

Fig. 4. RNase protection analysis of ϵ-globin RNA from peripheral blood and liver in staged transgenic embryos. The probes used are listed at the left and the size of protected products in nucleotides are indicated at the right. The RNA (100 ng) was prepared as described (*18*) and used for each protection analysis. Probes were from pSP64Mα (*19*), pSP64Mz (*19*), pSP65βh1 (*20*), pSP65Mε^{y} (*19*), and pSP64Hϵ$_{x}$ (*19*).

two mechanisms that control hemoglobin switching in humans: an autonomous mode, as exemplified by the ϵ gene, and a competitive mode, as illustrated by the γ to β switch. Autonomous and competitive mechanisms have been proposed for hemoglobin switching in the chicken (*15*). The transfected adult β-globin gene is only expressed in definitive cells of the chicken (*15, 16*) and thus appears to be autonomous in its developmental regulation. In contrast, the developmental regulation of the chicken embryonic ϵ genes appears to be competitive, because the restriction of its expression to primitive cells depends on the presence of the β-globin gene on the same plasmid (*15, 16*). Whereas the mechanisms of switching appear to be similar in these two species, the choice of the mechanisms with respect to specific genes is reversed. This is perhaps less surprising than it may seem, because at the divergence of aves and mammalia, only a single β-like globin gene existed (*17*). The duplications that gave rise to the human and chicken β-globin families occurred later and, therefore, independently in the two species.

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9. The hypersensitive fragments that constitute the μLCR and their nucleotide sequence coordinates (GenBank HUMBB accession no J00179) are as follows: Hind III 13769 to Nco I 13062; Bgl II 9218 to Hind III 8486; Hind III 5172 to Bal I 4608; Sph I 1702 to Pst I 1182. W. C. Forrester, U. Novak, R. Gelinas, M. Groudine, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5439 (1989).
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12. Transgenic mice with the human ϵ gene lacking the cis-linked LCR sequences have failed to show expression of the transgene at any developmental stage. Similar results were obtained in transgenic analyses of the human α-globin gene (*3*).
13. The mouse has two major functional β-like embryonic genes (βh1, ε^{y}) with different profiles of expression during the maturation of primitive erythroid cells, βh1 is maximally expressed before ε^{y}. The pattern of human ϵ-globin extinction in the blood of the staged embryos more closely resembled the ε^{y} profile than the βh1 profile. This implied functional relation between human ϵ and mouse ε^{y} is consistent with the evolutionary relation predicted from structural comparisons [A. Hill *et al.*, *J. Biol. Chem.* **259**, 3739 (1984)].
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19 June 1990; accepted 14 September 1990

Sequence-Specific DNA Binding by the c-Myc Protein

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While it has been known for some time that the c-Myc protein binds to random DNA sequences, no sequence-specific binding activity has been detected. At its carboxyl terminus, c-Myc contains a basic–helix-loop-helix (bHLH) motif, which is important for dimerization and specific DNA binding, as demonstrated for other bHLH protein family members. Of those studied, most bHLH proteins bind to sites that contain a CA– –TG consensus. In this study, the technique of selected and amplified binding-sequence (SAAB) imprinting was used to identify a DNA sequence that was recognized by c-Myc. A purified carboxyl-terminal fragment of human c-Myc that contained the bHLH domain bound in vitro in a sequence-specific manner to the sequence, CACGTG. These results suggest that some of the biological functions of Myc family proteins are accomplished by sequence-specific DNA binding that is mediated by the carboxyl-terminal region of the protein.

DESPITE A GREAT DEAL OF RESEARCH indicating that the *c-myc* oncogene functions in cell proliferation and differentiation, the molecular mechanisms of myc function remain unknown. Recent evidence suggests that *c-myc* may be involved in transcription, DNA replication or both (*1*), and thus might be expected to have a sequence-specific DNA-binding activity. However, although c-Myc has been shown to bind to random DNA sequences, no sequence-specific DNA-binding activity has been demonstrated (*2, 3*). c-Myc and other Myc family proteins contain a basic–helix-loop-helix (bHLH) domain, a conserved region that mediates DNA binding by other bHLH proteins (*4, 5*). The bHLH domain consists of the proposed HLH motif (*4*), which mediates homo- and heterooligomerization among certain bHLH family members (*6–8*). Immediately NH_2-terminal to the HLH region is a basic region that contacts DNA (*7, 8*). The c-Myc bHLH region and an immediately COOH-terminal leucine zipper motif (*1*) are essential for the cotransformation properties of myc (*9*). The binding sites that have been identified for other bHLH proteins contain a CA– –TG consensus (*4, 5,*

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