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Use of yeast artificial chromosomes (YACs) in studies of mammalian development: Production of β-globin locus YAC mice carrying human globin developmental mutants

(developmental regulation/transgenic mice/hereditary persistence of fetal hemoglobin/δβ-thalassemia)

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ABSTRACT **To test whether yeast artificial chromosomes (YACs) can be used in the investigation of mammalian development, we analyzed the phenotypes of transgenic mice carrying two types of β-globin locus YAC developmental mutants: (*i*) mice carrying a G → A transition at position −117 of the $^{A}\gamma$ gene, which is responsible for the Greek $^{A}\gamma$ form of hereditary persistence of fetal hemoglobin (HPFH), and (*ii*) β-globin locus YAC transgenic lines carrying δ- and β-globin gene deletions with 5′ breakpoints similar to those of deletional HPFH and δβ-thalassemia syndromes. The mice carrying the −117 $^{A}\gamma$ G → A mutation displayed a delayed γ- to β-globin gene switch and continued to express $^{A}\gamma$-globin chains in the adult stage of development as expected for carriers of Greek HPFH, indicating that the YAC/transgenic mouse system allows the analysis of the developmental role of cis-acting motifs. The analysis of mice carrying 3′ deletions *first* provided evidence in support of the hypothesis that imported enhancers are responsible for the phenotypes of deletional HPFH and *second* indicated that autonomous silencing is the primary mechanism for turning off the γ-globin genes in the adult. Collectively, our results suggest that transgenic mice carrying YAC mutations provide a useful model for the analysis of the control of gene expression during development.**

Molecular biological techniques have allowed the cloning and characterization of several mammalian developmental genes, and transgenic mice have been used extensively in the analysis of regulatory mechanisms controlling these genes. Although a powerful technique, gene transfer of recombinant constructs to generate transgenic mice suffers from several limitations. The size of the DNA molecule that can be injected has been constrained by limitations on the size that could be cloned and purified intact. Thus, presumably nonessential sequences are omitted in the design of constructs to be injected, and decisions are made about the regulatory relevance of the omitted sequences and the distances between cis control elements. To overcome these limitations, we have generated transgenic mice using purified yeast artificial chromosomes (YACs) (1). YACs offer two major advantages for the analysis of gene regulation. The first advantage is the insert size that can be contained within the YAC; the 100-kb to Mb size range allows the study of complete genes or multigene loci in the context of their native sequence environment. The second advantage is the ability to introduce site-directed mutations into sequences inserted in the YAC vector using the homologous recombination system of yeast harboring the YAC. We utilized a YAC containing the multigenic human β-globin locus for studies of developmental regulation of gene expression in transgenic mice (1). Our data show that the genes of the β-globin locus YAC (β-YAC) are correctly regulated during development in the mouse (1), thus demonstrating the usefulness of the YAC/transgenic mouse system.

In this work, we test whether YACs can be used for the analysis of developmental regulation by introducing mutations into the β-globin locus that affect cis-acting sequences known to cause aberrant developmental phenotypes. We demonstrate the usefulness of this system by introducing a point mutation that recreates the phenotype of the Greek $^{A}\gamma$ form of hereditary persistence of fetal hemoglobin (HPFH) and by analyzing the effects of deletions of the 3′ end of the β-globin locus. Our results indicate that mutant YACs can be used in the analysis of the cis control of developmentally regulated genes.

MATERIALS AND METHODS

Production of the −117 $^{A}\gamma^{m}$ YAC. A 5.4-kb *Ssp* I–*Sal* I fragment containing the −117 $^{A}\gamma^{m}$ gene was isolated from pUC19 $^{A}\gamma^{m}$ −117(+) (K.R.P., unpublished results) and ligated into *Sma* I–*Sal* I-digested and phosphatase-treated yeast integrating plasmid (YIP) vector pRS406 (Stratagene) to produce pRS406 −117 $^{A}\gamma^{m}$. Ten micrograms of pRS406 −117 $^{A}\gamma^{m}$ was linearized at a unique *Hin*dIII site in the $^{A}\gamma$ insert sequence and transformed into spheroplasted *Saccharomyces cerevisiae* strain AB1380 containing the YAC yneoβglobin (β-YAC; ref. 2). Transformants were selected for uracil prototrophy on complete minimal medium lacking uracil. YIP vector sequences and one copy of the duplicated $^{A}\gamma$ gene region were excised as follows. Yeast isolates were grown in complete minimal broth lacking tryptophan and lysine (to keep selection on the YAC arms) but containing uracil (to allow spontaneous excisions of the YIP vector via homologous recombination between the duplicated $^{A}\gamma$ gene regions). Yeast cells (10^{6}–10^{7}) were plated on 5-fluoroorotic acid plates to select for spontaneous YIP excision events (frequency of 10^{-4}–10^{-5}). Single colony isolates were further purified and retested for loss of the *URA3* gene and 5-fluoroorotic acid resistance.

Purification of the −117 $^{A}\gamma^{m}$ YAC. −117 $^{A}\gamma^{m}$ YAC DNA was purified essentially as described (1, 2) with the following modifications. Agarose plugs were not agitated during preparation to reduce shear of DNA molecules contained within the blocks (3). The entire 3- to 3.5-g gel slice was treated with agarase overnight. The supernatant (up to 4 ml) was passed through a molecular weight cut-off filter (Millipore; 100,000 nominal molecular weight limit) to concentrate the DNA

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Abbreviations: YAC, yeast artificial chromosome; HPFH, hereditary persistence of fetal hemoglobin; LCR, locus control region; HS, hypersensitive site; β-YAC, β-globin locus YAC; YIP, yeast integrating plasmid.

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essentially as described (1, 2). The use of a higher molecular weight cut-off filter increased the proportion of full-length YAC molecules recovered, and the greater volume of agarase-treated solution passed through the filter allowed more YAC DNA to be obtained. The DNA was diluted to 1 ng/μl in 10 mM Tris, pH 7.5/250 μM EDTA/100 mM NaCl and filtered as described (1) just prior to injection of fertilized mouse eggs. The maintenance of high salt throughout the purification and injection procedures was critical for assuring YAC integrity and was not found to be detrimental for the viability of the eggs. Transgenesis in founder animals was 14%.

Structural Analysis of the −117 $^{A}\gamma^{m}$ YAC. The presence of the −117 and $^{A}\gamma^{m}$ mutations in −117 $^{A}\gamma^{m}$ YAC transgenic mice was confirmed by DNA sequence analysis of a PCR-amplified fragment derived from tail blood DNA encompassing these two mutations. β-Globin locus insert–YAC vector junctions were detected by PCR analysis (1, 2). Intactness of the human β-globin locus in −117 $^{A}\gamma^{m}$ YAC transgenic mice was determined by detailed examination of the 140-kb *Sfi* I fragment encompassing most of the locus (Fig. 1*A*). Briefly, agarose plugs prepared from mouse liver cell suspensions were digested overnight with *Sfi* I, and the DNA was fractionated by pulsed-field gel electrophoresis as described (4). The gels were capillary blotted overnight to nylon membranes (Magnagraph NT; Micron Separations) in 10× SSC. Individual lanes were cut out, and each one was hybridized overnight to a different probe spanning the β-globin locus from 5′ hypersensitive site (HS) 3 to 3′ of 3′ HS 1 (Fig. 1). After washing, the membrane was reassembled and an autoradiograph or a phosphorimage (Molecular Dynamics PhosphorImager) was made. Different-sized *Sfi* I bands, including the intact 140-kb band, could be visualized, and approximate deletion endpoints in the nonintact bands were determined. The probes utilized included the following fragments (Figs. 1 and 5): 0.7-kb *Pst* I 5′ HS 3, 1.9-kb *Hin*dIII 5′ HS 2, 3.7-kb *Eco*RI ε gene, 2.4-kb *Eco*RI 3′ $^{A}\gamma$ gene, 1.0-kb *Eco*RV ψβ, 1.9-kb *Xba* I I, 0.8-kb *Xba* I L, 1.6-kb *Eco*RI–*Bgl* II N, 2.1-kb *Pst* I 5′ δ, 1.4-kb *Hpa* I 3′ δ, 0.9-kb *Eco*RI–*Bam*HI β gene exon II, 1.4-kb *Xba* I DF10 (3′ HS 1), 1.9-kb *Bgl* II HPFH 3, 0.5-kb *Hin*dIII H500, and 1.5-kb *Eco*RI–*Bgl* II HPFH 6. The ψβ, I, L, N, and 5′ δ probe template DNAs were gifts of N. P. Anagnou (University of Crete).

Measurement of Globin mRNA Synthesis. Total RNA isolation and RNase protection analysis were performed as described (1). Template DNAs for generating antisense RNA probes were the same except pT7 $^{A}\gamma^{m}$ (170) (5) was utilized to measure γ gene expression and the following additional templates were also included: pSP64 Hε$_x$, pSP6 βh1, pSP65 Mε^{y}, and pSP64 Mβ134 for measurement of human ε, mouse βh1, mouse ε^{y}, and mouse β^{maj} gene expression, respectively.

RESULTS

−117 $^{A}\gamma^{m}$ YAC Mice Have HPFH. The G → A substitution at position −117 of the distal CCAAT box of the $^{A}\gamma$ gene (6, 7) causes the Greek $^{A}\gamma$ form of HPFH. Individuals heterozygous for this mutation have from 10% to 20% fetal hemoglobin-containing $^{A}\gamma$ chains ($\alpha_2{}^{A}\gamma_2$; hemoglobin F, or Hb F), as well as β-globin production in cis (8).

The −117 $^{A}\gamma^{m}$ YAC was produced by homologous recombination after transformation of a β-YAC-containing yeast (1, 2) with a YIP carrying a portion of the $^{A}\gamma$ gene encompassing the −117 point mutation and a linked $^{A}\gamma^{m}$ mutation. The $^{A}\gamma^{m}$ mutation is a 6-bp deletion in the 5′ untranslated region of the $^{A}\gamma$ gene. The overall structure of the −117 $^{A}\gamma^{m}$ YAC derived from yeast was confirmed to be intact by hybridization of Southern blots of both standard and pulsed-field electrophoretic gels (data not shown). All −117 $^{A}\gamma^{m}$ YAC founders had increased γ-globin expression; at 6–8 weeks after birth the amount of γ mRNA as a percent of total human mRNA (ratio of γ to γ + β times 100%) ranged from 4% to 10% except for one founder that had 100% γ. Globin chain isoelectric focusing indicated that only $^{A}\gamma$ and β globin chains were produced in the adult −117 $^{A}\gamma^{m}$ YAC transgenics. Results from RNase protection analysis of seven founders, three of which were used to establish lines for further analysis, are shown in Fig. 2.

DNA from F_1 progeny of the three lines was subjected to structural analysis for the presence of left and right insert–YAC vector junction sequences by PCR (1, 2). Lines 2 and 7 had intact left and right insert–vector junctions; line 4 did not have either insert–vector junction. Agarose blocks containing total murine genomic DNA were prepared from liver cell suspensions of F_1 mice. Slices of each block were subjected to *Sfi* I restriction enzyme digestion, DNA was fractionated by pulsed-field gel electrophoresis, and the gel was analyzed by Southern blot hybridization. This method allows one to determine whether the 140-kb *Sfi* I fragment of the YAC is intact

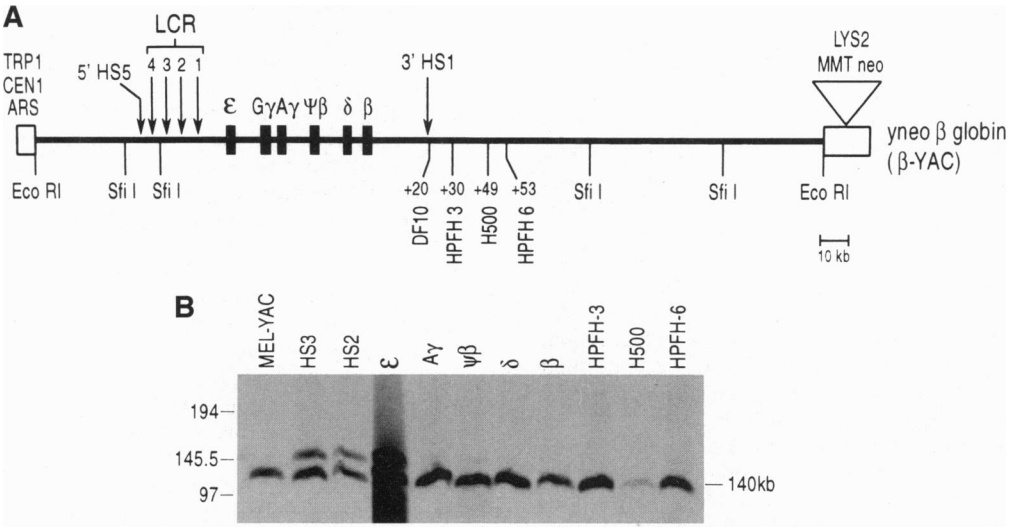

FIG. 1. (*A*) Diagram of the human β-YAC. The diagnostic 140-kb *Sfi* I fragment encompassing most of the β-globin locus spans between 5′ HS 3 and 5′ HS 4 to downstream of 3′ HS 1. (*B*) Structural analysis of −117 $^{A}\gamma^{m}$ YAC transgenic mouse line 2. *Sfi* I digestion, pulsed-field gel electrophoresis, and Southern blot hybridization of the individual lanes with the probe indicated above each lane of the autoradiogram were performed as described in *Materials and Methods*. MEL-YAC is a MEL cell line containing a single, intact copy of the wild-type β-YAC (4) probed with a 2.4-kb *Eco*RI 3′ $^{A}\gamma$ gene probe. LCR, locus control region.

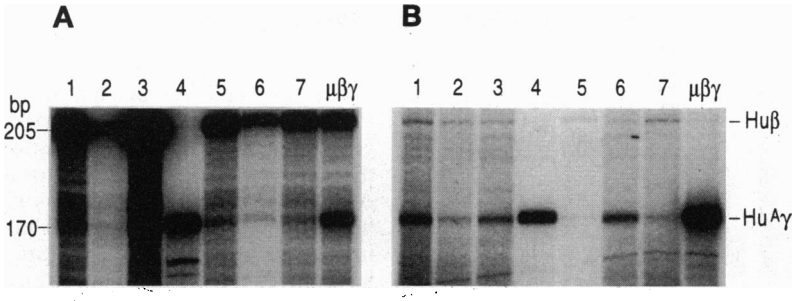

FIG. 2. −117 $^{A}\gamma^{m}$ YAC founder mice express γ-globin mRNA in adult blood. Total RNA isolated from the blood of seven founder mice was hybridized to human β and $^{A}\gamma$ antisense RNA probes. The founder number (1 to 7) is indicated above the autoradiograms. Total RNA derived from transgenic mice containing a μLCRβγ construct (μβγ) served as a positive control. The location of the human β (Huβ) and $^{A}\gamma$ ($Hu^{A}\gamma$) protected fragments are shown on the right, and their size in base pairs is indicated on the left. (*A*) RNase protection with both β and $^{A}\gamma$ RNA probes. (*B*) RNase protection with only the $^{A}\gamma$ RNA probe.

or if it contains deletions. Line 2 (Fig. 1*B*) has an intact 140-kb *Sfi* I fragment containing the entire β-globin locus and a second 165-kb *Sfi* I fragment containing sequences that only hybridized with the 5′ HS 3, 5′ HS 2, and ε gene probes. Line 4 shows a single, smaller than wild-type fragment (83 kb) that contains 5′ HS 3 through δ but is deleted between the δ- and β-globin genes (data not shown). Line 7 displays two *Sfi* I fragments, both larger than the 140-kb wild-type size, 195 and 310 kb, indicative of the loss of one or both *Sfi* I sites (data not shown). The β-globin locus is intact in both bands from 5′ HS 3 to the HPFH 3 probe downstream from 3′ HS 1.

−117 $^{A}\gamma^{m}$ YAC Mice Display a Delayed γ to β Switch. Representative examples of the changes in expression of murine and human genes in wild-type β-YAC and −117 $^{A}\gamma^{m}$ YAC transgenic mice are shown in Fig. 3. Human ε and murine ε^{y} are restricted to embryonic erythropoiesis; human β and murine β are restricted to liver and bone marrow erythropoiesis. Human γ, like its murine orthologue βh1, is expressed in the yolk sac; however, unlike βh1, which is restricted to the yolk sac, human γ continues to be expressed in the liver stage of erythropoiesis. γ expression is switched off after birth, and no γ mRNA signal can be detected by RNase protection after postnatal day 7.

In contrast to the wild-type β-YAC line (Fig. 3*B*), the −117 $^{A}\gamma^{m}$ YAC lines display a delayed γ- to β-globin gene switch (Fig. 3*C*). Thus, in the β-YAC line, the switch from γ- to β-globin gene expression occurs in the blood at about 14 days of development and is complete shortly after birth; the γ mRNA levels are ≈10% of total human globin at day 17 of development and <0.1% in the adult state. In contrast, in the blood of the −117 $^{A}\gamma^{m}$ YAC line, γ gene expression remains elevated through 17 days of development, making up ≈40% of the total human mRNA on that day. γ-Globin gene expression stabilizes at about 8% of total human globin gene expression 3 weeks after birth. A delayed γ to β switch was previously observed in transgenic mice carrying a LCR −117 γβ minilocus construct (9).

Normally, only adult murine globin is expressed in liver erythropoiesis. In the wild-type β-YAC line, there is both human γ- and β-globin gene expression in the liver; γ gene mRNA is maximal at 12 days (30% of total human globin) and decreases thereafter (Fig. 4*A*). In the −117 $^{A}\gamma^{m}$ YAC line, human γ-globin mRNA is 93% of the total human globin mRNA in the day 11 fetal liver and 70% at day 13 (Fig. 4*B*). The level of mRNA falls to 55% at day 15 and is still at 35% at day 17. Thus, the γ to β switch in the −117 $^{A}\gamma^{m}$ YAC line is much more gradual compared with that in the wild-type β-YAC mice.

δ- and β-Globin Gene Deletions Do Not Result in Activation of γ Gene Expression. Human mutations that delete the δ and β genes and various lengths of 3′ sequence produce phenotypes of HPFH or δβ-thalassemia. In deletional HPFH, ≈20 to >100 kb of the 3′ end of the β-globin locus is removed, including the δ- and β-globin genes. Individuals heterozygous for such mutations usually have 25–35% fetal hemoglobin in their peripheral blood (8). In δβ-thalassemias, there is usually continued Hb F production in the adult but at levels substantially lower than those of deletional HPFH. The structure of deletions observed in δβ-thalassemia differs from those observed in deletional HPFH.

Four lines with the 5′ end of the β-globin locus intact, but carrying 3′ β-YAC deletions resembling those of HPFH or δβ-thalassemia deletional mutants, have been obtained from

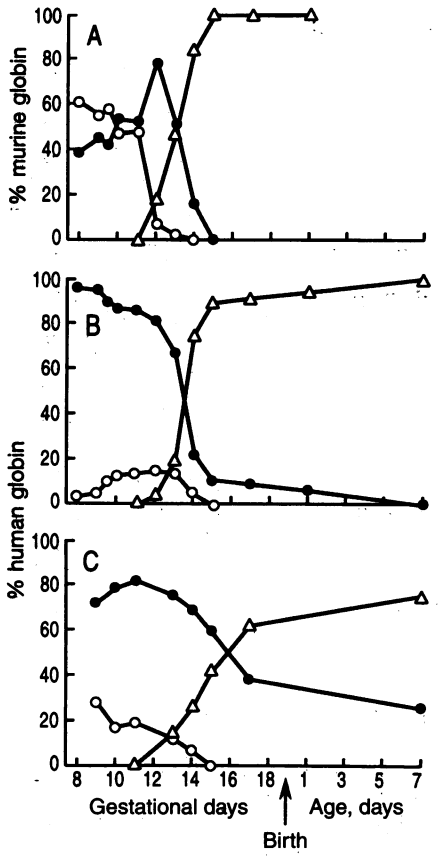

FIG. 3. The γ- to β-globin gene switch is delayed in the blood of −117 $^{A}\gamma^{m}$ YAC transgenic mice. Globin mRNA is expressed as percentage of total human globin mRNA (*y* axis) for each day of development (*x* axis). (*A*) Switch of the endogenous murine β-like globin genes. ○, ε^{y}; ●, βh1; △, β^{maj}. (*B*) Human β-like globin gene switching in wild-type β-YAC transgenic mice. ○, ε; ●, $^{A}\gamma$; △, β. (*C*) Human β-like globin gene switching in −117 $^{A}\gamma^{m}$ YAC line 2 transgenic mice. Symbols are as for *B*.

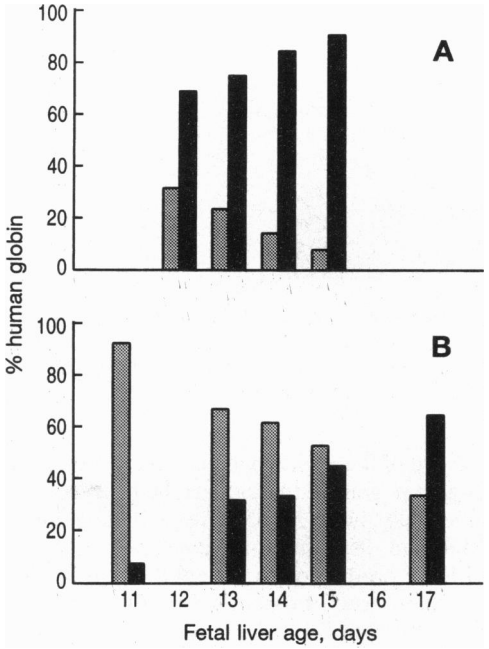

FIG. 4. Human γ-globin mRNA production in murine fetal liver erythropoiesis. γ and β mRNAs were quantitated as described in the legend to Fig. 3. The *y* axis of both panels is the level of either γ or β mRNA as a percentage of total human globin mRNA for a given day. The stippled bars are $^{A}\gamma$ and the black bars are β. (*A*) Wild-type β-YAC transgenic mouse fetal liver. (*B*) −117 $^{A}\gamma^{m}$ YAC line 2 transgenic mouse fetal liver.

spontaneous rearrangements of wild-type β-YAC transgenes (ref. 1; Fig. 5*A*). Several probes were used to resolve the deletion breakpoints between the $^{A}\gamma$- and β-globin genes (Fig. 5*B*). Lines Δ1 and Δ2 had deletion breakpoints just 3′ to the ψβ pseudogene—i.e., very close to the 5′ breakpoint of previously described HPFH mutants (10, 11). In contrast to the human deletional HPFH mutants, these lines had low (about 1%) levels of γ mRNA (ratio of γ to mouse α times 100%, not corrected for copy number). Line Δ3 had a deletion breakpoint between $^{G}\gamma$ and $^{A}\gamma$—i.e., a breakpoint previously observed in $(^{A}\gamma\delta\beta)^{0}$-thalassemia. In this human mutation, high levels of $^{G}\gamma$ gene expression are characteristic. In contrast we could not detect $^{G}\gamma$ mRNA in the blood of adult mice of line Δ3. Line Δ4 had a breakpoint just 5′ to or within the δ-globin gene that had been previously described in a δβ-thalassemia mutant; humans heterozygous for a mutation having this 5′ breakpoint have increased γ expression (12); we could not detect any γ mRNA in the adult transgenic mice of this line.

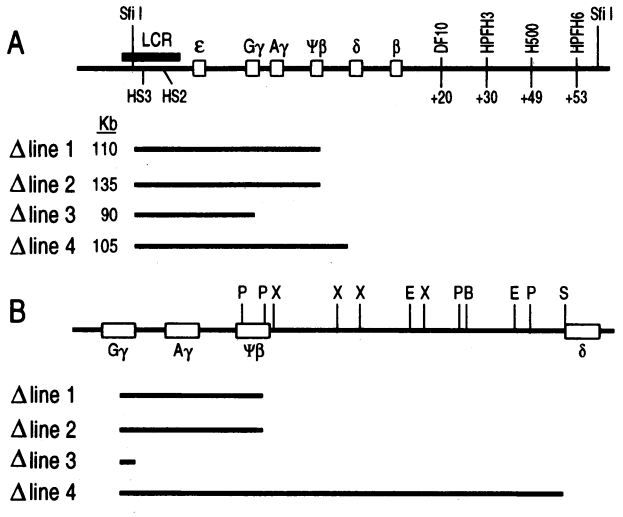

FIG. 5. Structures of 3′ deletion β-YACs. The deletion lines (Δ 1–4) are indicated on the left below each schematic diagram; the size of the *Sfi* I fragment for each 3′ deletion β-YAC line is indicated next to the line number in *A*. The right end of each line is the approximate 5′ deletion breakpoint. (*A*) Gross structure of the 3′ deletion β-YACs. The probes utilized in this analysis are shown above the line, except 5′ HS 3 and 5′ HS 2, which are shown below the line. (*B*) Fine mapping of the 3′ deletion β-YAC endpoints. The region from $^{A}\gamma$ to δ has been expanded from *A*. Restriction enzyme sites are shown above the line: B, *Bgl* II; E, *Eco*RI; P, *Pst* I; S, *Sal* I; X, *Xba* I. Probes used in this analysis included γ, ψβ, I, L, N, and δ (see *Materials and Methods*).

DISCUSSION

In this work, we examined whether transgenic mice containing YACs can be used for studying developmental regulation. As a test, we introduced a point mutation into the β-YAC that causes the Greek form of HPFH characterized by the presence of 10–20% $^{A}\gamma$-globin-containing fetal hemoglobin in the blood of heterozygous individuals. While adult transgenic mice carrying a wild-type β-YAC totally lack γ-globin expression, transgenic mice carrying the −117 $^{A}\gamma^{m}$ YAC continued to produce $^{A}\gamma$ globin in the adult. The reproduction of a human developmental mutation in the YAC transgenic mouse shows that this system can be used for analysis of the developmental control of human loci. Substitutions can be made in putative regulatory sequences in the context of whole loci, and the effect of such mutations can be analyzed in transgenic mice carrying the whole locus under examination. Such analysis is less informative with single genes or other short constructs from which regulatory sequences (or sequences of unknown function) are omitted.

The continuation of $^{A}\gamma$ expression in adult individuals carrying the −117 $^{A}\gamma$ HPFH mutation has been explained by two mechanisms (6–9). First, the mutation may increase γ promoter strength, allowing the mutant γ promoter to interact with adult stage-specific trans-activating factors. Second, the mutation may eliminate or inhibit binding of a repressor. The delayed γ to β switch during fetal development in the transgenic mice supports the second hypothesis. In the mouse, the adult erythroid environment is established as soon as the fetal liver stage of erythropoiesis starts. If the −117 $^{A}\gamma$ mutation makes the $^{A}\gamma$ promoter capable of interacting with adult stage-specific erythroid factors, the level of $^{A}\gamma$ gene expression should have been constant throughout the murine fetal liver stage of erythropoiesis. The gradual decline of $^{A}\gamma$ gene expression is more consistent with the gradual appearance of a γ gene repressor during fetal development.

A significant problem of the YAC transgenic mice is the presence of YAC rearrangements. Such rearrangements result in spontaneous deletions, the analysis of function of which may provide insight on the developmental control of the locus under investigation. This is illustrated in our study. Spontaneous deletions of the 3′ end of the β-globin locus produced four mutants with 5′ breakpoints resembling those of deletional HPFH, $(^{A}\gamma\delta\beta)^{0}$-thalassemia, or $(\delta\beta)^{0}$-thalassemia mutants. Not one of these β-YAC mutants was associated with the abundant γ gene expression that is characteristic of human mutations having similar 5′ breakpoints. These results provide insights on the mechanism of γ gene activation in HPFH and δβ-thalassemia and on the mechanism of γ gene silencing during development.

For several years, the differing phenotypes of HPFH and δβ-thalassemia have been a puzzle. In both conditions the δ- and β-globin genes are deleted, but in HPFH γ-globin production is abundant and hematological findings are normal, whereas in δβ-thalassemia γ-globin production is low, resulting in deficient hemoglobin content of the red cell and a phenotype of thalassemia (8). Two major hypotheses have been

forwarded to interpret the two phenotypes. One hypothesis proposes that a regulatory element responsible for γ gene silencing is located between the $^{A}\gamma$- and the δ-globin genes (13). In HPFH the deletion removes the γ gene silencer, while in δβ-thalassemia it does not; the differing 5′ breakpoint of the deletion thus determines the phenotype. As mentioned earlier, the 5′ breakpoints of two of our β-YAC deletion mutants are very close to those of HPFH mutants; however, the β-YAC deletion mutants did not have abundant γ gene expression, which is the hallmark of deletional HPFH. It is thus unlikely that deletion of a silencer located in the ψβ–δ intergenic region is responsible for the HPFH phenotype. The second hypothesis attributes the phenotype of HPFH and δβ-thalassemias to the action of cis enhancers juxtaposed to the γ gene as a result of the deletion (10, 14, 15). Our results indirectly support this hypothesis.

Two mechanisms have been proposed to account for β-like globin gene switching: gene silencing and gene competition. The best example of gene silencing is that of the ε-globin gene (16, 17), the expression of which is totally restricted to embryonic erythropoiesis. The competitive mechanism (18–23) is based on the assumption that the promoters of globin genes compete, at any developmental time, for interaction with the LCR. A considerable amount of evidence supports the competitive mechanism of silencing of the β-globin genes in the embryonic stage of development (18, 20, 21, 24). Both competitive and autonomous models have been proposed to explain the turning off of the γ-globin genes in the adult stage of development (5, 20, 25). The autonomous control is thought to be achieved through the action of cis-acting silencer elements located in the proximal and distal γ-globin gene promoters (5, 25). The competitive model assumes that a preferential interaction between the β-globin gene and the LCR in the adult stage of development underlies the turning off of the γ-globin genes (18, 20). As shown here, the absence of δ and β genes in the 3′ deletion β-YAC mutants is not associated with continuation of γ gene expression in the adult stage of development. This result suggests that autonomous silencing rather than gene competition is the primary mechanism for turning off γ gene expression in the adult.

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