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Genetics

Germ-line transmission and developmental regulation of a 150-kb yeast artificial chromosome containing the human β-globin locus in transgenic mice

(microinjection/pulsed-field gel electrophoresis/primer extension/Southern blot)

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ABSTRACT **Sequential expression of the genes of the human β-globin locus requires the formation of an erythroid-specific chromatin domain spanning >200 kb. Regulation of this gene family involves both local interactions with proximal cis-acting sequences and long-range interactions with control elements upstream of the locus. To make it possible to analyze the interactions of cis-acting sequences of the human β-globin locus in their normal spatial and sequence context, we characterized two yeast artificial chromosomes (YACs) 150 and 230 kb in size, containing the entire β-globin locus. We have now successfully integrated the 150-kb YAC into the germ line of transgenic mice as a single unrearranged fragment that includes the locus control region, structural genes, and 30 kb of 3′ flanking sequences present in the native locus. Expression of the transgenic human β-globin locus is tissue- and developmental stage-specific and closely follows the pattern of expression of the endogenous mouse β-globin locus. By using homology-directed recombination in yeast and methods for the purification and transfer of YACs into transgenic mice, it will now be feasible to study the physiological role of cis-acting sequences in specifying an erythroid-specific chromatin domain and directing expression of β-globin genes during ontogeny.**

The human β-globin gene locus contains five functional genes that are sequentially expressed during human development. Expression of the genes (the embryonic ε, the fetal $^{A}\gamma$ and $^{G}\gamma$, and the adult δ and β) is erythroid- and developmental stage-specific (reviewed in ref. 1). While sequences immediately flanking the genes are sufficient to direct low-level tissue and stage-specific expression, elements 5′ to the β-globin gene family are required for high-level transcription of all β-like globin genes (2–13).

Structural analyses of the human β-globin locus have determined that the entire human β-globin gene region is DNase I-sensitive and early replicating throughout erythroid development (8, 14, 15). Four DNase I-hypersensitive sites (5′HSs 1–4), located 6–18 kb 5′ to the ε-globin gene, have been designated the locus control region (LCR) (10). Naturally occurring deletions of this region result in transcriptional silencing of all of the downstream β-like genes (6–8, 16). The presence of the LCR in transgenic constructs results in high-level erythroid-specific expression of linked β-like genes. In addition, downstream to the locus, there are HSs 22 and 100 kb 3′ to the adult β-globin gene. Putative control elements >70 kb 3′ to the human β-globin gene that contain enhancer-like elements have also been described (11, 15, 17). Thus, sequences spanning a region of 150–200 kb have been implicated in the control of the human β-globin gene family.

Previous studies have examined the regulation of globin genes with constructs consisting of selected genes and elements of the LCR. Due to the size constraints of plasmid and cosmid vectors, large portions of the native human β-globin locus were necessarily deleted. To analyze the sequences controlling globin expression in a more physiologic chromosomal context, we have characterized a 150-bp and a 230-kb yeast artificial chromosome (YAC) containing the entire human β-globin locus (18). We now report the successful transfer of the 150-kb YAC, carrying the LCR and the β-globin locus in their native context, into the germ line of two transgenic mouse lines following microinjection of purified β-globin YAC DNA. We find that the human locus is expressed in a tissue- and stage-dependent manner paralleling the expression of the endogenous mouse β-globin locus.

MATERIALS AND METHODS

YAC Purification. Individual isolates of *Saccharomyces cerevisiae* strain AB1380 carrying a 150-kb YAC (A201F4) containing the entire human β-globin locus were grown up and the high molecular weight DNA was purified in agarose blocks, loaded onto preparative gels, placed in a CHEF apparatus (Bio-Rad), and subjected to pulsed-field gel electrophoresis (PFGE) as described (18). The YAC DNA slice was excised from the preparative gel, and the gel slice was equilibrated overnight in 50 mM Tris, pH 6.5/10 mM EDTA/100 mM NaCl, digested with β-agarase according to the manufacturer's protocol (New England Biolabs), and centrifuged at 1200 × *g* for 5 min to precipitate undigested agarose. The YAC was concentrated in dialysis tubing in sucrose and dialyzed against 10 mM Tris, pH 7.4/0.16 mM EDTA for 48 hr. To assess the integrity of the purified YAC DNA, preparations were subjected to PFGE and Southern hybridization (18). Solutions containing YAC DNA at 2–4 ng/μl were used for microinjection.

Analysis of Transgenic Mice. The purified A201F4 YAC was microinjected into fertilized oocytes from B6SJL mice as described (19). Transgenic animals were identified by using embryo, placental, or tail clip DNAs, as appropriate, and analyzed by PCR, Southern blot hybridization, and PFGE (18).

RNA was prepared from yolk sacs or livers of embryos at postcoital days 8.5, 10.5, 11.5, 12.5, and 14.5, and from adult spleens 6 days following intraperitoneal injections of phenylhydrazine (0.04 mg/g) on days 1–3 (20). RNA was purified as described (21).

Primer extension assays were performed essentially as described (22, 23), using primers for mouse ε^{y}, β^{h1}, and β^{major}; human β-globin primers (24); and human ε and γ primers with the sequences 5′-GCCTCTTCCACATTCATCTTGCTCCACAGGCTAGTGACG-3′ and 5′-TCACCTTGCCCCACAG-

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Abbreviations: HS, DNase I-hypersensitive site; LCR, locus control region; PFGE, pulsed-field gel electrophoresis; YAC, yeast artificial chromosome.

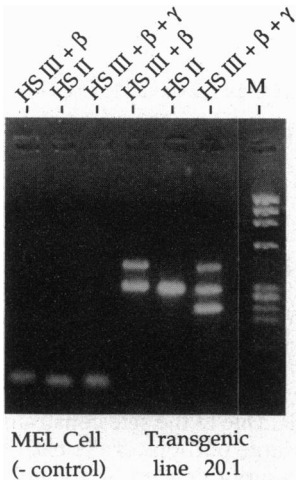

FIG. 1. PCR analysis of DNA from a mouse erythroleukemia (MEL) cell line (negative control) and transgenic founder line 20.1. For each, from left to right: lane 1, coamplification of 5'HS 3 (420 bp) and human β-globin gene (310 bp); lane 2, 5'HS 2 (310 bp); lane 3, 5'HS 3, human β and γ (234 bp). Lane M contained *Hae* III-digested φX174 DNA (size markers). PCR primer pairs were as follows: HS3, 5'-GGAGTCAAGGCACTTGCCCCTAGCTGG-3' and 5'-GGAATCATTCTGTGGATAAAGG-3'; HS2, 5'-CTGTGTGTCTCCATTAGTGACCTCCC-3' and 5'-TGATGCCGTTTGAGGTGGAGTTTTA-3'; human γ, 5'-ATGGCGTCTGGACTAGGAGC-3' and 5'-GCAAATATCTGTCTGAAACGGTTCC-3'; human β, 5'-TACGTAAATACACTTGCAAAGGAGG-3' and 5'-TTTGAGGTTGCTAGTGAACACAGTT-3'.

GCTTGTGATAGTAGCCTTGTCC-3', respectively. Intensities of bands were analyzed with a PhosphorImager (Molecular Dynamics).

RESULTS

Characterization of Transgenic Lines. Genomic DNA from founder mice was first screened by PCR using primer pairs from 5'HS 3, 5'HS 2 and the human γ- and β-globin genes (Fig. 1). To verify results obtained by PCR and to map the transgenic sequences in more detail, all the pups were further analyzed with Southern blot hybridization of *Eco*RI-digested genomic DNA. Two founders, 20.1 and 20.7, carried sequences from the LCR and all the genes of the human β-globin region. As 20.7 was a female that could not be successfully bred, further mapping was pursued only for mice generated from 20.1.

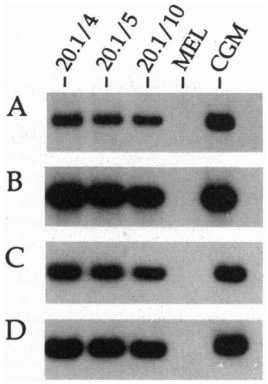

FIG. 2. Southern analysis of *Eco*RI-digested DNA from three 20.1 F_1 mice, mouse erythroleukemia cells (MEL, negative control), and the human CGM1 cell line. The probes used are shown in Fig. 3, and the four probes displayed here are 3.3 RI (*A*), ε (*B*), γ (*C*), and RK29 (*D*).

Southern analysis of several transgenic F_1 animals from 20.1 established the presence of all the probe sequences tested with the exception of markers for the left (trp) arm of the pYAC4 vector, and the 1.8 RI probe located 5' of the LCR (Figs. 2 and 3). The hybridized fragments were identical in size from line 20.1 and human genomic DNA. PhosphorImager analysis revealed that the intensity of the line 20.1 DNA hybridization signal with each probe was half that of CGM1, the diploid cell line from which the YAC was prepared. Hence, the 20.1 line carries a single copy of the human β-globin genomic insert.

To assess the integrity of the human β-globin locus in line 20.1, agarose blocks containing high molecular weight DNA were digested with *Kpn* I, or with *Sal* I and *Sfi* I, and subjected to PFGE and Southern blot analysis (Fig. 4). The

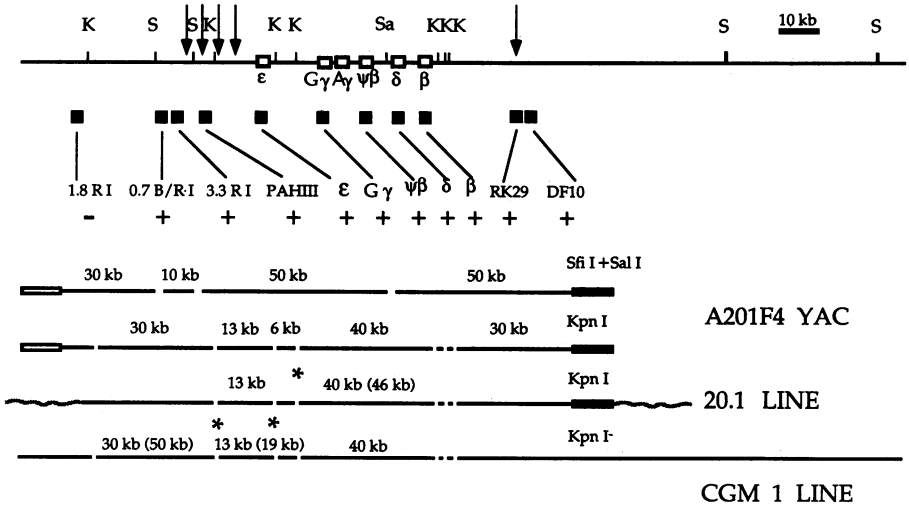

FIG. 3. Map of the human β-globin gene region of A201F4 YAC, transgenic line 20.1, and CGM1 cell line. Top line indicates the positions of the genes (open squares) and the HSs (arrows). The restriction sites of *Kpn* I (K), *Sfi* I (S), and *Sal* I (Sa) are shown. The positions of probes used to map the human locus in Southern hybridization are represented by the solid boxes and their presence (+) or absence (−) in transgenic line 20.1 is indicated. The line drawings below represent the restriction fragment lengths (kb) of the A201F4 YAC, 20.1 transgenic line, and human CGM1 cell line after digestion with the enzymes shown. The solid and open rectangles at either end of the A201F4 and 20.1 map depict, respectively, the *ura* and *trp* vector arms of the YAC vector. Wavy lines in the map of transgenic line 20.1 represent flanking mouse genomic sequences. Restriction sites at which partial cleavage was observed in the mouse and human genomic DNA (Fig. 4) are identified by asterisks above the sites, and the length of the partially digested fragments are shown in parentheses.

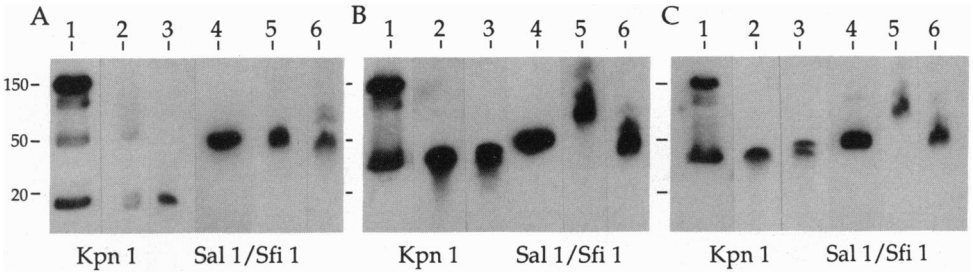

FIG. 4. PFGE analysis of A201F4 YAC sequences in transgenic line 20.1. DNA samples from A201F4 YAC (lanes 1 and 4), CGM1 cell line (lanes 2 and 5), and transgenic line 20.1 (lanes 3 and 6) were digested with *Kpn* I or with *Sal* I and *Sfi* I as indicated. Gels were transferred to nylon membranes and hybridized with probes for the ε (*A*), δ (*B*), or β (*C*) globin genes. Because of the large difference in the copy number of the human sequences between yeast strain A201F4 and the CGM1 and 20.1 DNA samples, autoradiograms from 1- to 5-day exposures are shown.

human ε-globin gene resides on the expected 13-kb *Kpn* I fragment in line 20.1 (Fig. 4*A*, lanes 1–3). Additional bands present in the yeast and human DNA lanes are the products of partial digestion. In the *Sal* I/*Sfi* I digest, the ε-globin gene in line 20.1 is also located on the correct 50-kb fragment (Fig. 4*A*, lanes 4–6). Human δ- and β-globin genes are normally located on a 40-kb *Kpn* I fragment. All three DNAs contain this fragment (Fig. 4 *B* and *C*, lanes 1–3). The larger fragments in the YAC, as well as the 46-kb fragment in line 20.1, are due to partial digestion. The fragments hybridizing with δ- and β-globin gene probes in *Sal* I/*Sfi* I digests of CGM1 DNA (Fig. 4 *B* and *C*, lane 5) are larger than those seen in yeast (lane 4) and line 20.1 (lane 6). This is due to the presence of an *Sfi* I site 70 kb 3′ to the adult β-globin gene in the human genomic DNA and a more proximal *Sal* I or *Sfi* I site in the flanking mouse DNA in line 20.1 (Fig. 3). Additional mapping with the DF10 and 3.3 RI probes (data not shown) shows that the A201F4 YAC has integrated in a single, continuous segment without internal rearrangement.

Analysis of Human β-Globin Locus Expression. To determine whether expression of the transgenic human β-globin locus was restricted to erythroid tissue, primer extension analysis was performed on total RNA from adult F_1 tissues following treatment of the animals with phenylhydrazine (Fig. 5). Expression of the human β-globin RNA was only seen in blood and spleen samples (Fig. 5). Low-level β-globin mRNA seen in the kidney is probably due to residual blood contamination of this tissue.

To establish the pattern of expression of the transgenic human β-globin locus during development, RNA was analyzed by primer extension (Figs. 6 and 7). At day 8.5, the first human gene expressed was the fetal γ-globin gene. In day 10.5 yolk sac, the percentage of γ-globin mRNA, while the ε-globin mRNA level increased, and this trend continued on day 11.5. At day 12.5, expression of the ε-globin gene had largely been switched off. There is an abrupt shift to β-globin gene expression that exceeds the level of γ-globin mRNA. Human β-globin expression continued to increase at day 14.5 as the dominant globin mRNA. Quantitation by PhosphorImager analysis revealed that the ratio of adult mouse to adult human β-globin gene activity per gene copy was 1.35. Thus, the expression level of the single, integrated human β-globin locus was comparable to that of the endogenous murine β-globin locus. The pattern of ε- and β-globin gene activity followed the expression of the mouse embryonic and adult genes, respectively. However, expression of the human γ-globin gene preceded ε-globin gene expression at day 8.5 and persisted throughout the fetal stage. In adult transgenic animals, we occasionally detected low-level human fetal gene expression (Fig. 7). This may be a consequence of the extreme erythroid hyperplasia observed after treatment with phenylhydrazine.

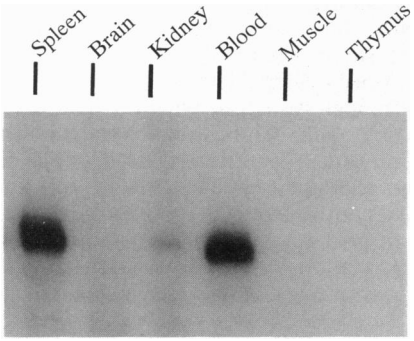

FIG. 5. Tissue specificity of human β-globin gene expression. RNA from adult spleen, brain, kidney, blood, muscle, and thymus was purified from tissues harvested on day 6 following treatment of transgenic mice with phenylhydrazine. Primer extension analysis for globin sequences was performed on 5 μg of total RNA.

DISCUSSION

To study human globin gene regulation within the context of an intact chromosomal locus, we have purified a 150-kb YAC

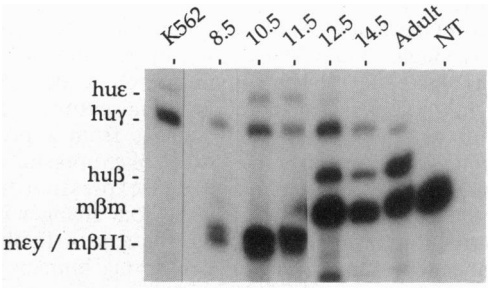

FIG. 6. Developmental expression of the transgenic human β-globin locus. Transgenic embryos were identified by PCR. RNA was isolated at days 8.5, 10.5, and 11.5 from yolk sacs and at days 12.5 and 14.5 from fetal liver and simultaneously annealed to primers for the human ε, γ, and β globin mRNAs and for the mouse embryonic (ε^y and β^{h1}) and adult β^{major} globin mRNAs. The sizes of primer extension products from the different globin mRNAs are as follows: human ε (huε), 124 bp; human γ (huγ), 110 bp; human β (huβ), 95 bp; mouse β^{major} (mβm), 85 bp; mouse ε^y and β^{h1} (mεy and mβH1), 75 bp. Mouse embryonic primers annealed more quantitatively in the buffer described by Krakowsky *et al.* (23); however, spurious bands appeared at later time points. For this reason, day 8.5, 10.5, and 11.5 yolk sac RNAs were annealed to all the mouse and human β-like primers according to the Krakowsky protocol, whereas RNAs from later time points were annealed in formamide buffer (22). The first lane contains RNA from a human K562 erythroleukemia cell line that expresses ε- and γ-globin, and the rightmost lane contains RNA from a nontransgenic (NT) mouse. The developmental stage of mice at which transgenic RNA samples were purified is indicated above each lane.

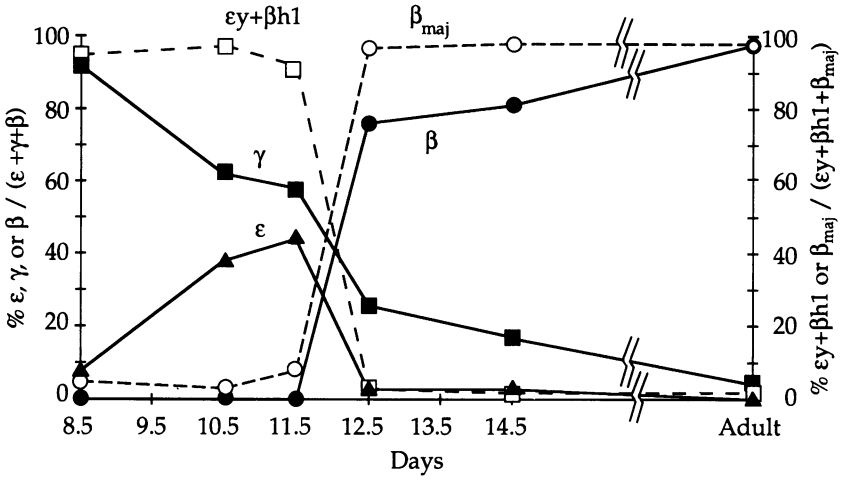

FIG. 7. Pattern of human ε-, γ-, and β-globin gene expression in transgenic line 20.1 during development. Mouse and human β-like RNAs were quantitated with a PhosphorImager and corrected for the specific activity of each primer. The level of RNA of each human gene, calculated as the fraction of total human ε-, γ-, or β-globin RNA at each stage, is plotted in solid lines and symbols. The expression level of each gene reflects the average of several experiments. Expression of the individual mouse β-like globin genes was calculated similarly and is plotted in dashed lines and open symbols. The developmental time points at which RNA was harvested appear at the bottom.

that includes all the sequences that have been shown to be involved in β-globin gene control, and introduced the YAC into transgenic mice. The integrated YAC in line 20.1 has lost the left (trp) vector arm as well as 10–20 kb of 5′ flanking region upstream of the LCR. The remaining 130 kb of the YAC, containing the β-globin cluster and its control region, was integrated as a single intact fragment. The expression of this transgenic human β-globin locus is tissue- and stage-specific. Interestingly, while the pattern of expression of the human ε- and β-globin genes closely follows that of the endogenous mouse embryonic and β^{major} genes, respectively, the human γ-globin gene is activated earlier than the human ε gene in the mouse, rather than the reverse order as in humans. This pattern of human γ-globin activity has also been described in transgenic lines where the human β-globin locus was transferred following ligation of two contiguous cosmids (25).

Erythroid development of most mammals, including mice, involves a single switch from embryonic to adult globin chain synthesis. However, in the simian branch, a separate set of fetal β-globin genes, designated γ-globin, evolved (26). Human fetal globin genes probably arose from a primordial globin gene with an embryonic pattern of expression (3). The delay in the onset of human γ-globin expression from the embryonic to the fetal stage evolved from changes in either trans-acting factors, or cis-acting sequences flanking the γ-globin genes, or both. In mice carrying human γ-globin genes in the absence of the LCR, γ-globin genes are activated in embryonic yolk sac and fetal liver (3). Therefore, sequences sufficient to direct embryonic activation are present within or flanking the γ-globin genes. This suggests that separate regulatory mechanisms must have evolved to control early activation and silencing of human embryonic globin genes and later silencing of the fetal gene. The development of transgenic mice carrying the intact human β-globin locus makes it feasible to test whether evolutionary changes in cis- or trans-acting factors are primarily responsible for the fetal pattern of human γ-globin gene expression.

Several features of β-globin gene regulation have emerged from studies carried out with individual and multigene constructs containing fragments of the human β-globin locus. In general, high-level, copy number-dependent expression of human β-globin genes is observed only when elements of the LCR are present in the transgene (9, 12, 24, 25, 27–34). In addition, the native gene order and distance from the LCR are probably important for correct timing and levels of gene activity (27, 28, 35). Interactions between the genes themselves, such as competition for trans-acting factors, appear to play a role in developmental switching (24, 27, 28, 31). The LCR appears to function as an erythroid-specific enhancer in gene-transfer experiments, containing binding sites for a variety of ubiquitous and erythroid-specific transcription factors (36–38). In addition, the LCR has an essential role in determining the overall structure and replication pattern of the erythroid-specific chromosomal domain encompassing the locus (8, 15). Nevertheless, despite intensive study, the inability to analyze the genes of the human β-globin locus in their normal genomic context has made it difficult to precisely characterize the complex mechanisms underlying tissue- and stage-specific expression.

Recently, several groups have transferred YAC DNA into cell lines and fertilized oocytes (39–48). The use of YACs as transgenic constructs confers several important advantages over the transfer of plasmid and cosmid constructs in globin studies. (*i*) The transfer of these YACs will permit the analysis of an intact regulatory domain encompassing the entire locus. (*ii*) Efficient techniques for homologous recombination in yeast make site-directed mutagenesis of cis-acting sequences in the human β-globin locus readily feasible (49). (*iii*) The low copy number of YACs microinjected reduces the probability of sequences integrating in tandem arrays that may create expression artifacts or α/β-globin chain imbalance with potential adverse effects on the transgenic animals (50). (*iv*) Naturally occurring mutations, such as silent β-thalassemia, whose functional effect has only been inferred from genetic studies in families, may now be tested. (*v*) Transgenic mice carrying the intact human β-globin locus are also a good model system in which to test drugs, such as butyrate analogs, that induce human fetal globin expression and ameliorate the severity of sickle cell disease and β-thalassemia in human patients (51–53). (*vi*) The ability to transfer YACs into mice will permit the identification of genes by molecular complementation and the study of structure–function relationships of very large genes whose size has previously prevented such analysis.

Note Added in Proof. Peterson *et al.* (54) have recently published similar studies using a β-globin YAC described in ref. 18.

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