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Use of yeast artificial chromosomes (YACs) for studying control of gene expression: correct regulation of the genes of a human beta-globin locus YAC following transfer to mouse erythroleukemia cell lines.

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Immunology. In the article "Prolonged and effective blockade of tumor necrosis factor activity through adenovirus-mediated gene transfer" by J. Kolls, K. Peppel, M. Silva, and B. Beutler, which appeared in number 1, January 4, 1994, of *Proc. Natl. Acad. Sci. USA* (**91,** 215–219), the following correction should be noted. In the legend to Fig. 3, the text for *A* and *B* was transposed. The correct legend should read as follows: Fig. 3. (*A*) cfu of *L. monocytogenes* present in the liver and spleen of animals pretreated with the control adenovirus or the TNF inhibitor adenovirus. Error bars represent standard deviation among three animals treated with each virus. (*B*) Appearance of the liver and spleen of representative mice inoculated with 4×10^3 cfu of *L. monocytogenes* 3 days after receiving 10^9 pfu of adenovirus coding for β-galactosidase (control) or TNF inhibitor. Note that the liver and spleen are of normal appearance in the control animal and are whitish in color in animals that received the inhibitor adenovirus.

Developmental Biology. In the article "Use of yeast artificial chromosomes (YACs) for studying control of gene expression: Correct regulation of the genes of a human β-globin locus YAC following transfer to mouse erythroleukemia cell lines" by Kenneth R. Peterson, Galynn Zitnik, Clare Huxley, Christopher H. Lowrey, Andreas Gnirke, Kathleen A. Leppig, Thalia Papayannopoulou, and George Stamatoyannopoulos, which appeared in number 23, December 1, 1993 of *Proc. Natl. Acad. Sci. USA* (**90,** 11207–11211), the authors request that the following correction be noted. There is an error in the legend of Fig. 1. It reads "containing three copies (*a* and *c*) or one copy (*b* and *d*)." It should read "containing three copies (*a* and *b*) or one copy (*c* and *d*)." The authors apologize for any confusion this may have produced.-

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Developmental Biology

Use of yeast artificial chromosomes (YACs) for studying control of gene expression: Correct regulation of the genes of a human β-globin locus YAC following transfer to mouse erythroleukemia cell lines

(developmental regulation/locus control region/DNase I sensitivity/somatic cell hybrids/gene transfer)

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ABSTRACT **We demonstrate that transfer of a yeast artificial chromosome (YAC) containing 230 kb of the human β-globin locus into mouse erythroleukemia cells by fusion results in correct developmental regulation of the human β-like globin genes. Additionally, we show that early after hybrid formation, human embryonic ε- and fetal γ-globin genes are coexpressed with the adult β gene but that after 10–20 weeks in culture, globin gene expression switches to predominantly adult. Thus, in contrast to shorter gene constructs, the globin genes of the β-globin locus YAC are regulated like the chromosomal globin genes. These results indicate that transfer of YACs into established cell lines can be used for the analysis of the developmental control of multigenic and developmentally regulated human loci.**

Transfections of established cell lines with recombinant gene constructs have been useful in identifying cis-acting regulatory elements. However, there have been size constraints on how large a DNA sequence can be introduced into a cell by existing methods. An ideal approach would be to introduce an entire multigenic locus into a cell, thereby retaining the proper spatial context of genes, flanking control regions, and cis-acting sequences. In this work we show that normal regulation of the globin genes of a 248-kb yeast artificial chromosome (YAC) containing 230 kb of the human β-globin locus is established following transfer into mouse erythroleukemia (MEL) cells. Furthermore, the globin genes of the YAC display a temporal switch from fetal γ- to adult β-globin expression and they respond to trans-acting stimuli that activate the chromosomal γ genes. Our findings imply that transfer of YACs into established cell lines can be used in the analysis of the developmental control of multigene loci.

The functional genes of the human β locus are arrayed 5′ to 3′ in the order in which they are expressed during development, 5′-ε-^{G}γ-^{A}γ-δ-β-3′. Upstream of the ε gene is the locus control region (LCR), defined as a set of four erythroid-specific and developmentally stable DNase I-hypersensitive sites (designated 5′ HS 1 to 5′ HS 4) (1–3) that confer high-level, copy number-dependent, integration site-independent expression of globin genes in erythroid cells (3). Another developmentally stable HS, termed 3′ HS 1, is present ≈20 kb 3′ to the β gene (1–3). Several cis control sequences of the β locus have been identified by transfecting recombinant globin gene constructs into cell lines, especially MEL cells. MEL cells express only adult murine globins at low, but detectable levels and can be induced by a variety of agents to terminally differentiate and produce high levels of globin. MEL cells transfected with human β gene constructs linked to the LCR or to individual HSs display high-level expression of the human β-globin gene (4–6). When human fetal globin genes are transferred into MEL cells, either alone or in a β-locus cosmid, they are inappropriately expressed (7–10), indicating that perhaps sequences outside the transfected DNA fragments are relevant to the developmental regulation of fetal globin genes. In the experiments reported here, we used a 248-kb YAC (11, 12) containing 230 kb of the human β-globin locus and flanking sequences; 82 kb comprise the actual human β-globin locus from 5′ HS 4 to 3′ HS 1, and there are about 40 kb of additional sequence 5′ to HS 4 and about 110 kb 3′ to the β globin gene.

MATERIALS AND METHODS

Hybrids were generated by fusion of G418-resistant, YAC-containing murine L cells (adherent phenotype) with G418-sensitive MEL cells (nonadherent phenotype). Approximately 5×10^6 MEL cells and 2.5×10^6 L cells were washed free of serum, mixed, and centrifuged down in one pellet and fused as described (13). After 48 hr of culture, nonadherent cells were centrifuged and resuspended in fresh Iscove's modified Dulbecco's medium with 20% fetal bovine serum and containing active G418 sulfate (Geneticin, GIBCO) at 0.9 mg/ml in a new tissue culture flask. Subsequently, every 2 or 3 days the nonadherent cells were poured into a new tissue culture flask, in order to select for the nonadherent phenotype. Parental MEL cells were killed by the G418 and parental L cells were removed each time the cells were transferred into a new flask. After about 10 days to 2 weeks in culture, clumps of vigorously growing cells were observed. The cells were induced for 4 days in medium containing 2% (vol/vol) dimethyl sulfoxide (Sigma). Globin assessment at the protein level was accomplished by staining cell smears with γ- or β-globin-specific monoclonal antibodies as described (13).

Pulsed-field gel electrophoresis (PFGE) conditions were 1% SeaKem Gold agarose (FMC), 0.5× TBE, (1× is 89 mM

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Abbreviations: YAC, yeast artificial chromosome; MEL, mouse erythroleukemia cells; LCR, locus control region; HS, hypersensitive site; PFGE, pulsed-field gel electrophoresis.

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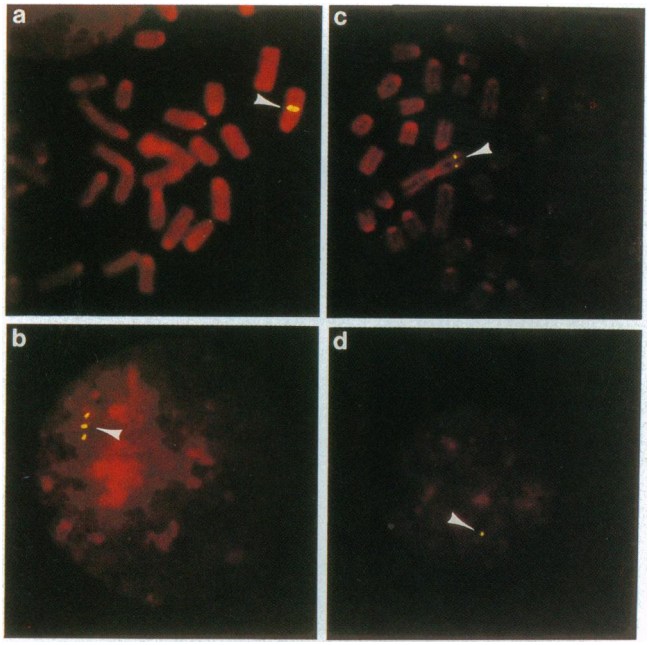

FIG. 1. Fluorescence *in situ* hybridization of $^{G}\gamma^{A}\gamma\psi\beta\delta\beta$ cosmid to L—MEL cell hybrids containing three copies (*a* and *c*) or one copy (*b* and *d*) of a human β-globin locus YAC. (*a* and *c*) The arrows point to the fluorescein signal resulting from hybridization of cosmid cCH-1 to propidium iodide-stained metaphase chromosomes. (*b* and *d*) Three distinct fluorescein signals (in *b*) and one signal (in *d*) are observed in interphase nuclei.

Tris/89 mM boric acid/2 mM mM EDTA), 14°C, 24 hr, with a 14-sec pulse time. Agarose plugs for PFGE analysis were prepared from single-cell suspensions as described (12) and DNA contained in plugs was digested with *Sfi* I. Southern blots and hybridization were performed by standard methods (14). For RNase protection, previously described techniques and probes were used (15). For DNase I hypersensitivity assays, aliquots of nuclei were digested at DNase I concentrations ranging from 0 to 5.0 μg/ml, in 0.5-μg/ml increments (16), and genomic DNA was digested with *Eco*RI. The probes used in these assays are described in the legend of Fig. 2.

RESULTS

Transfer of a β-Globin Locus YAC into MEL Cells. To allow selection of somatic cells containing the β-locus YAC, a neomycine-resistance gene conferring G418 resistance on mammalian cells was introduced into the right arm of pYAC4 vector sequence. Attempts to introduce the purified β-YAC directly into MEL cells by electroporation or microinjection were unsuccessful. Therefore, we employed an alternative strategy of first injecting the β-YAC into mouse L cells and then transferring the β-YAC-containing chromosome into MEL cells by fusion. The YAC was introduced into the L-cell line L A-9 by microinjection (12). PCR analysis showed that the left and right vector–insert junctions were intact, RecA-assisted restriction endonuclease cleavage showed that the entire 230-kb insert was present, *Sfi* I digestion coupled with PFGE and Southern blotting demonstrated the presence of a 140-kb *Sfi* I fragment encompassing the β-globin locus, and other Southern blot analyses confirmed the presence of an intact β-globin gene (12).

To introduce the β-YAC into MEL cells we transferred the β-YAC-containing chromosome from the mouse L cells into MEL cells by fusion. Two L-cell isolates (12), Iβ6, with a single copy of the intact YAC, and Iβ5, with three copies of the intact YAC, were used for fusion with MEL lines 179 and

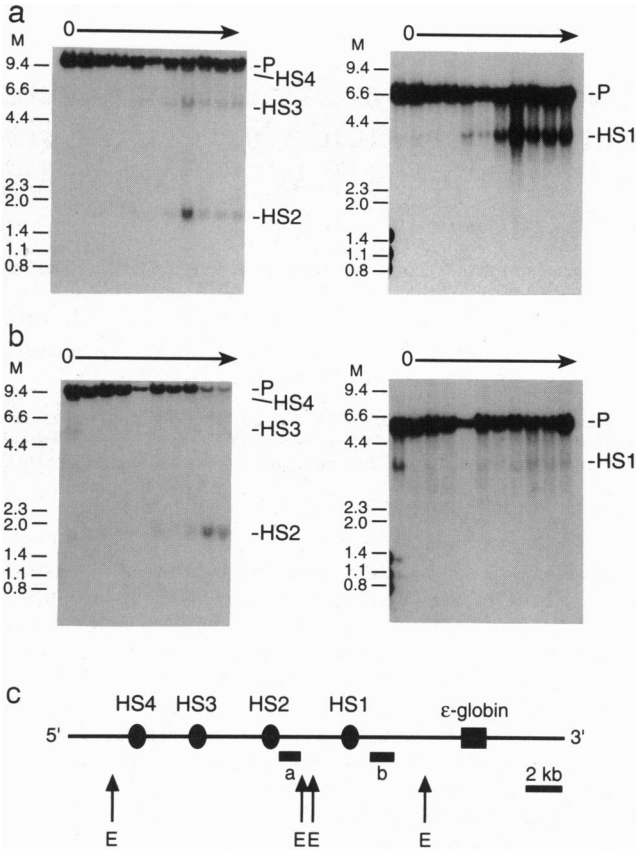

FIG. 2. Formation of LCR HSs. (*a*) Analysis of hybrid pool D shows the formation of 5′ HSs 1–4. (*b*) Analysis of the Iβ6 L-cell line shows the formation of a strong 5′ HS 2, similar to that seen in hybrid D. The expected locations of 5′ HSs 3 and 4 are indicated but neither was formed. A weak 5′ HS 1 is present. (*c*) Map of the β-globin LCR showing the locations of the 5′ HSs, *Eco*RI sites, and the probes used in these experiments. *Eco*RI digestion yields a 10.4-kb fragment containing 5′ HSs 2–4 and a 6.5-kb fragment containing 5′ HS 1. Southern blot analysis utilized a 1.2-kb *Bgl* II–*Hin*dIII probe to detect 5′ HSs 2–4 (probe a). A 1.3-kb *Sph* I–*Ssp* I probe was used to detect 5′ HS 1 (probe b). E, *Eco*RI sites; P, parental band; M, molecular size markers (kb). Arrows indicating increasing concentrations of DNase I from 0 to 5.0 μg/ml.

585. G418-resistant, nonadherent L—MEL hybrid cells were collected; parental cells were selected against by their G418 sensitivity (MEL cells) or adherence (L cells). Four hybrid pools, A and B (containing three copies of the YAC) and C and D (containing one copy of the YAC) were produced. A and C were products of fusion with MEL 179, while B and D were from fusions with MEL 585. *Sfi* I digestion, PFGE, and Southern blotting analysis demonstrated that the human β-globin locus was intact (data not shown). Additional Southern blot analyses showed correct size fragments for the LCR, the ε, $^{A}\gamma$, and β genes, and the neomycin-resistance gene. Thus, the hybrids contained intact β-YAC sequences, in the appropriate copy number.

To determine whether the three YAC inserts in hybrid B were clustered at a single site or dispersed throughout the genome, fluorescence *in situ* hybridization was performed (17) with cosmid probe cCH-1, which contains 39 kb of the human β-globin locus from the $^{G}\gamma$ gene through the β gene. In all 50 metaphase cells scored from hybrid B, the signals from cosmid cCH-1 were present on both chromatids of a single chromosome in each metaphase. Examination of hybrid B interphase nuclei demonstrated three distinct hybridization signals in close proximity, which is consistent with the presence of three copies of the β-globin YAC in this cell line and suggests that

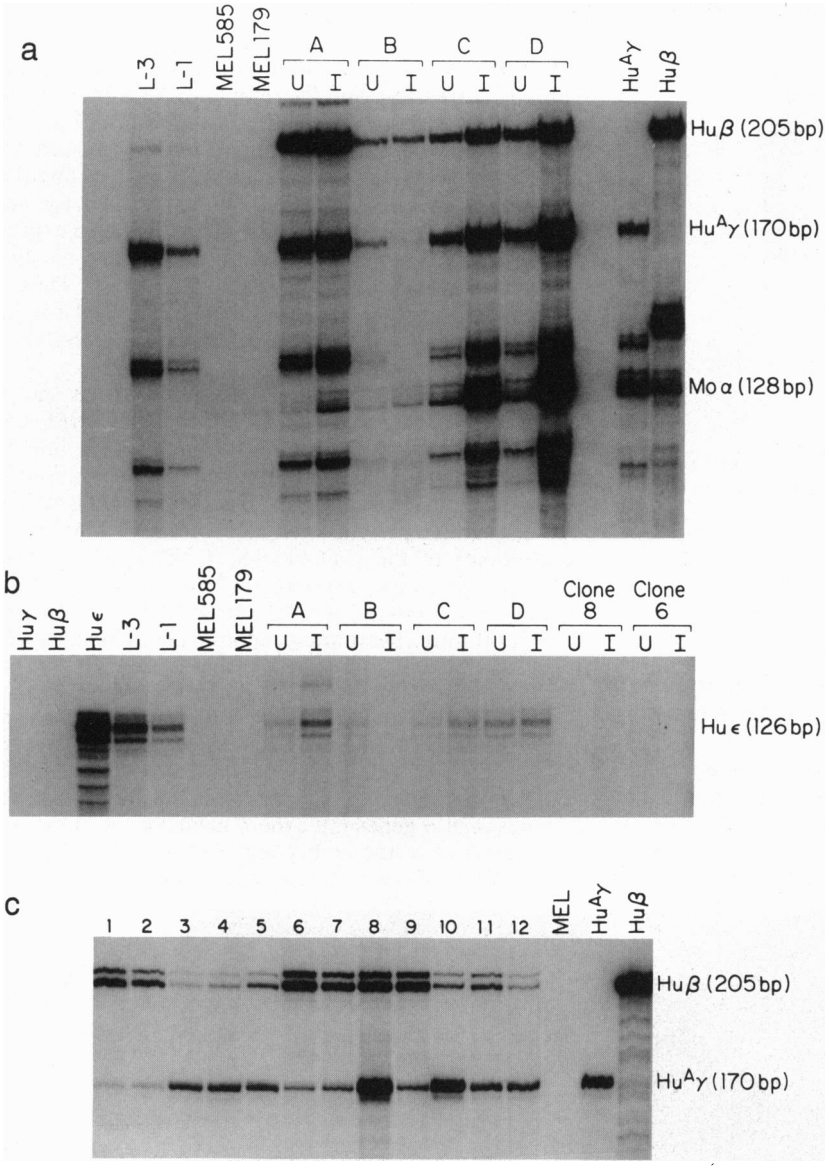

FIG. 3. Human globin expression in β-YAC L—MEL hybrids. Human (Hu) γ- or β- or mouse (Mo) α-globin mRNA have protected fragments shown on the right, along with the size of the protected fragments in base pairs. Hybrids A and B contain three copies of the β-YAC, whereas C and D contain one copy. L3 and L1 are the Iβ6 and Iβ5 β-YAC L-cell transfectants (12) used in the fusions. Letters U and I indicate uninduced cells and cells induced by dimethyl sulfoxide. (*a*) Notice the presence of approximately equal amounts of human γ and β mRNA in all hybrids. There is predominantly human γ mRNA in L1 and L3 β-YAC transfectants. (*b*) Presence of human ε mRNA in hybrids and L cells. (*c*) Human globin gene expression in 12 single-cell clones.

these copies are arranged in tandem fashion (Fig. 1*b*). As a control we hybridized cosmid cCH-1 to interphase cells from hybrid D; a single signal was found, consistent with a single copy of the β-globin YAC in these cells (Fig. 1*d*).

We examined the ability of characteristic HSs in the human β-globin LCR to form in the β-YAC-containing cell lines. As shown in Fig. 2, the four HSs that are characteristic of the LCR in erythroid cells, 5′ HSs 1–4, were formed in L—MEL hybrid pool D. When assayed in L-cell line Iβ6, a strong 5′ HS 2 was demonstrated, similar to that observed in hybrid pool D. The 5′ HS 1 was formed but exhibited a decreased sensitivity to DNase I digestion. The 5′ HSs 3 and 4 were not formed. Our results in the L-cell line are consistent with a previous report (18). The formation of these HSs in line Iβ6 is most likely related to the ability of the β-YAC-containing L-cell lines to transcribe the γ- and ε-globin genes and, to a lesser extent, the β-globin gene (Fig. 3 *a* and *b*).

Initial ε-, γ-, and β-Globin Expression and Subsequent Switch to Predominant β-Globin Expression from the Genes of the β-YAC. All hybrids maintained their erythroid cell phenotype as evidenced by their ability to terminally differentiate after chemical induction with dimethyl sulfoxide [compare lanes U (uninduced) and I (induced) of Fig. 3]. For analysis of β-globin gene expression, 28 days after fusion, total RNA was prepared from pellets of the cultured hybrid pools and analyzed by RNase protection using human ε, ^{A}γ, and β or mouse α antisense RNAs as probes. All four hybrid pools expressed human γ-, β-, and ε-globin mRNAs (Fig. 3 *a* and *b*). ε gene expression was minor; the exposure time of the autoradiograph shown in Fig. 3*b* was 2 weeks, in contrast to an exposure time of 16 hr for the autoradiograph shown in Fig. 3*a*. In all four pools, the γ gene accounted for a significant proportion of human globin gene mRNA (Fig. 3*a*). Thus, early after hybrid formation, the human globin genes displayed fetal as well as adult globin expression in spite of the adult environment of the MEL cell.

To examine whether the phenotype of the hybrids changed over time, hybrid pools C and D (containing a single β-YAC) were followed in culture for 17 weeks. Hybrid C switched off γ expression between 8 and 12 weeks (Fig. 4*a*). Hybrid D, in which 50% of the human globin mRNA at 8 weeks was γ, expressed <5% γ mRNA after 17 weeks in culture (Fig. 4*a*). ε mRNA could not be detected after the sixth week in culture. Staining the hybrids with fluorescent anti-γ or anti-β monoclonal antibodies showed that only rare cells were γ-positive after 3–4 months in culture (Fig. 5). These results indicated that, in contrast to the globin gene fragments (7–9) or β-locus cosmids (ref. 10 and G.S., unpublished data) used before, the globin genes of the β-locus YAC display correct developmental regulation following transfer into MEL cells. The

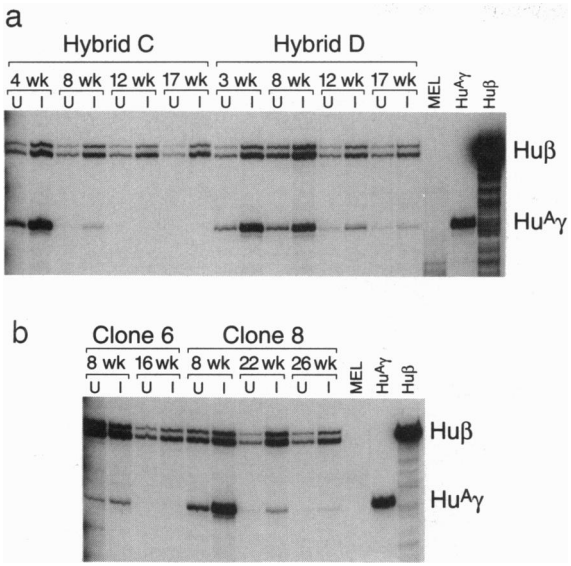

FIG. 4. Globin gene switching in β-YAC L—MEL hybrids. (*a*) Switching during culture of hybrids C and D of Fig. 3*a*. (*b*) Switching during culture of clones 6 and 8 of Fig. 3*c*.

human genes initially display a fetal-like globin program but subsequently switch to adult globin expression, thus mimicking the *in vivo* developmental γ-to-β switch.

Subcloning experiments were done to exclude the possibility that any changes in globin gene expression during culture were due to a population selection artifact. Twelve single-cell subclones were produced from hybrid pool D on culture day 39 and were analyzed 2 weeks later. As shown in Fig. 3*c*, there was heterogeneity in human globin expression among the subclones. Subclones 1 and 2 showed predominantly β expression, subclones 3 and 4 predominantly γ expression, and the remaining subclones displayed approximately equal levels of γ and β expression. Two of these subclones, clone 6, which initially showed high β expression, and clone 8, which initially showed about equal levels of γ and β expression, were cultured for several weeks (Fig. 4*b*). Clone 6 switched to exclusive β expression between 8 and 16 weeks of culture, whereas clone 8 switched between 22 and 26 weeks in culture. Thus, as in the parental hybrid, the hybrids of single-cell origin displayed a switch from γ to β expression as culture time advanced.

Response to Transactivation. To test whether the globin genes of the YAC respond to transregulation, Iβ6 and Iβ5 cells were fused with GM979 cells, a MEL line which coexpresses adult and embryonic globins and transactivates the chromosomal human γ-globin genes following fusion with adult human lymphoblasts (19). Hybrid E of Fig. 6 contains three copies of the β-globin locus YAC, while hybrid F contains one copy. Hybrid E expressed predominantly γ-globin [$\gamma/(\gamma+\beta)$ ratios ranged from 0.85 to 0.87] after 9–13 weeks in culture. There was predominant γ-globin expression in hybrid F after 12 and 16 weeks in culture [$\gamma/(\gamma+\beta)$ ratios ranged from 0.89 to 0.97]. Thus, the genes of the β-locus YAC displayed a predominant fetal globin expression program at a time when their counterparts which had been transferred into MEL cells had switched to predominant or exclusive adult globin expression (Fig. 4). These results suggest that like the chromosomal γ genes (19), the γ genes of the β-locus YAC are responsive to the embryonic/fetal trans-acting environment of the GM979 erythroleukemia line.

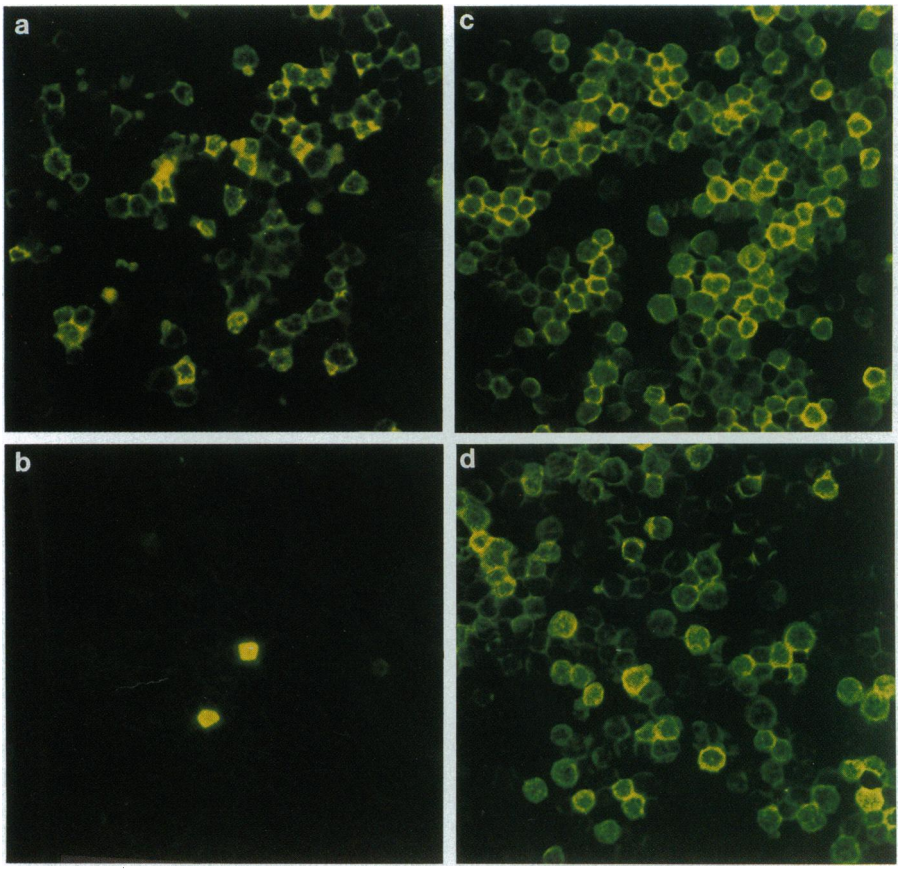

FIG. 5. Cytocentrifuged smears of induced β-YAC L—MEL hybrids were fixed and labeled with anti-γ- and anti-β-specific antibodies. Three weeks after fusion β-YAC/MEL hybrids contain a high proportion of γ (*a*) or β (*c*) globin-positive cells. Two-and-a-half months later γ-globin is present only in rare cells (*b*), whereas a large number of cells continue to express β-globin (*d*).

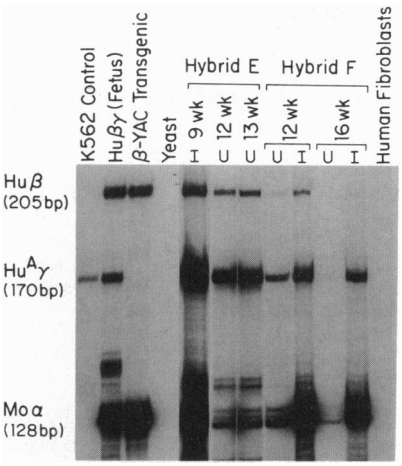

FIG. 6. Human γ- and β-globin gene expression in β-YAC L—GM979 cell hybrids. Protected fragments and their sizes are as in Fig. 3. E and F are hybrids containing three or one copy of the β-YAC, respectively. U denotes uninduced cells; I denotes dimethyl sulfoxide-induced cells. γ-Globin gene expression predominates over β-globin gene expression even after 3–4 months in culture. Hu, human; Mo, mouse.

DISCUSSION

Analysis of the control of gene expression by the use of transfer to established cell lines is based on the production of recombinant constructs containing the genes of interest and suspected regulatory sequences. This approach has allowed delineation of several cis-acting sequences immediately flanking the globin genes (reviewed in ref. 20). In the case of multigenic loci or when regulatory elements are placed far away from the structural genes, this approach creates problems, since size limitations in construct production constrain the amount of sequence one can transfer, obliging one to produce artificial recombinants by linking sequences normally located distant to the genes of a locus. During the production of such recombinants, assumptions are made about the importance of sequences that are omitted and about the relevance of factors such as gene order or the distance between genes and regulatory elements. For example, in the case of the β-globin locus, analysis of function of the LCR is based on production of artificial constructs made by combining LCR sequences with individual globin genes or with cosmids containing several contiguous globin genes (4–6, 21–23). Even when large constructs are produced by ligation of two cosmids (23), likely regulatory sequences such as the 3′ HS 1 are omitted. Furthermore, no construct can accommodate the β-locus chromatin domain as defined by DNase I sensitivity studies (1–3, 24). Our results, showing correct developmental regulation of the globin genes of the β-locus YAC, imply that transfer of β-locus YACs into MEL cells can be used for the analysis of regulatory elements of the β locus without distorting normal chromosomal organization.

The finding that human ε-, γ-, and β-globin transcription occurs in the L cells prior to fusion with MEL cells suggests that the fetal globin genes of the YAC are preactivated in the L cells. This can explain the initial γ and ε gene expression in the βYAC L—MEL cell hybrids. However, subsequently fetal gene expression switches off and there is only, or predominantly, adult human globin gene expression. Such temporal changes in expression are not observed when fetal globin genes on smaller constructs are transferred into MEL cells. Thus, when the $^{A}\gamma$, δ, and β genes linked to an LCR cassette on a cosmid construct are transferred to MEL cells, the γ gene is expressed at very high levels (ref. 10 and G.S., unpublished data) and there is no γ-to-β switch during MEL cell propagation in culture. The temporal switch of the globin genes of the β-YAC is thus similar to that observed in neonatal cells. Also, as we show here, the transferred YAC responds to trans stimuli after fusion with GM979 cells, which contain fetal-like trans-acting elements. The β locus of the YAC thus behaves distinctly differently than the gene fragments, underscoring the importance of distant regulatory elements on globin gene expression.

An implication of these results is that transfer of β-locus YACs into MEL cells can be used for the analysis of regulatory elements of the β locus without the distortion of locus organization necessitated by the size constraints of usual recombinant constructs. The system we describe here may be applicable to the analysis of regulatory elements of other multigenic loci or very large genes. YAC transfer into differentiating cell lines of the proper lineages makes studies of genes in their native environment an attractive alternative to transfecting simple recombinant molecules into cells.

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