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Established Epigenetic Modifications Determine the Expression of Developmentally Regulated Globin Genes in Somatic Cell Hybrids

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Somatic cell hybrids generated from transgenic mouse cells have been used to examine the developmental regulation of human γ-to-β-globin gene switching. In hybrids between mouse erythroleukemia (MEL) cells and transgenic erythroblasts taken at various stages of development, there was regulated expression of the human fetal γ and adult β genes, reproducing the in vivo pattern prior to fusion. Hybrids formed from embryonic blood cells produced predominantly γ mRNA, whereas β gene expression was observed in adult hybrids and a complete range of intermediate patterns was found in fetal liver hybrids. The adult environment of the MEL cells, therefore, did not appear to influence selective transcription from this gene complex. Irradiation of the embryonic erythroid cells prior to fusion resulted in hybrids containing only small fragments of donor chromosomes, but the pattern of gene expression did not differ from that of unirradiated hybrids. This finding suggests that continued expression of *trans*-acting factors from the donor erythroblasts is not necessary for continued expression of the human γ gene in MEL cells. These results contrast with the lack of developmental regulation of the cluster after transfection of naked DNA into MEL cells and suggest that epigenetic processes established during normal development result in the gene cluster adopting a developmental stage-specific, stable conformation which is maintained through multiple rounds of replication and transcription in the MEL cell hybrids. On prolonged culture, hybrids that initially expressed only the γ transgene switched to β gene expression. The time period of switching, from ~10 to >40 weeks, was similar to that seen previously in human fetal erythroblast × MEL cell hybrids but in this case bore no relationship to the time of in vivo switching. It seems unlikely, therefore, that switching in these hybrids is regulated by a developmental clock.

Differential gene expression underlies many of the events of cellular determination and differentiation which characterize development. Of the model systems used for the study of gene regulation during normal human development, the α- and β-globin gene clusters are among the best characterized (16, 38). Differential expression of these genes results in the sequential production of different hemoglobins at embryonic, fetal, and adult stages of ontogeny. An understanding of this system may therefore give valuable insights into general regulatory mechanisms directing the changes of gene activity during development.

The human globin genes are arranged in the order in which they are expressed in development. The α cluster on chromosome 16 consists of three functional genes, 5′-ζ-α2-α1-3′, and the β complex on chromosome 11 contains five functional genes, 5′-ε-^{G}γ-^{A}γ-δ-β. Both sets of genes are under the control of sequence elements lying several kilobases upstream, termed HS-40 in the α complex (18) and the locus control region (LCR) in the β complex (15). From both clusters, there is a change in gene expression at ~6 to 8 weeks development, from ζ to α and ε to ^{G}γ and ^{A}γ. In addition, the β cluster undergoes a further switch in the perinatal period from ^{G}γ and ^{A}γ to δ and β. A critical issue in understanding the developmental stage-specific expression of the globin genes is whether the complement of transcription factors present at the time of transcription is the sole determinant of differential gene expression. Selective gene expression may be established by interactions earlier in erythroid cell differentiation, resulting in differential accessibility of the genes for transcription; in this case, the *trans*-acting factors present during transcription may be permissive rather than selective.

The majority of models proposed to explain the details of hemoglobin switching have assumed, tacitly or explicitly, that developmental stage-specific transcription factors, interacting with the globin gene promoters, are the essential determinants of differential gene expression (11, 12, 41). Indeed, there are several lines of experimental evidence indicating that such *trans*-acting factors do play a role in the developmental regulation of these genes (3, 4, 8, 22, 25). Putative factors have been identified (5, 14, 20, 21), but in no case has it been demonstrated that they play a selective role in globin gene switching.

Less attention has been given to the possibility that epigenetic modifications of the globin gene clusters are important determinants of developmental gene expression. Mechanisms involving the establishment of a pattern of gene expression by one set of factors, which is then stably maintained by other factors, have been demonstrated to play major roles in *Drosophila* development (6, 9, 24, 28, 33, 35) and in yeast cells (2, 23). Epigenetic alterations acquired during the developmental or differentiation history of a cell result in stable, somatically heritable changes and may be brought about by mechanisms which alter DNA methylation, topology, chromatin structure, heterochromatinization, or nuclear compartmentalization (27, 34). These initial changes must be established by the interaction of *trans*-acting factors with DNA sequences, but once the changes are established, these factors need not persist (i.e., a hit-and-run effect) and a stable expression pattern may be maintained by permissive competence factors through subsequent cell divisions.

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Studies of somatic cell hybrids involving erythroid cells can be interpreted as showing that epigenetic changes acquired during an erythroid cell's developmental history may be important in globin gene switching. Hybrids in which a human chromosome 11 was transferred to mouse erythroleukemia (MEL) cells by cell fusion produced large amounts of human globin RNA and protein. Determination of which human globin gene was expressed appeared to depend on the cellular and ontogenic origin of the human chromosome, in that when fetal erythroblasts were the donor cells, the hybrids initially produced fetal γ chains, whereas when the chromosome was derived from adult cells, only the adult β chain was produced (13, 32).

Prolonged culture of the human fetal erythroblast × MEL cell hybrids resulted in a switch from γ gene to β gene expression over a time course of 10 to 40 weeks. The time of in vitro switching was inversely related to the gestational age of the fetuses at the time of cell fusion, leading to the suggestion that switching might be regulated by a developmental clock (32). As chromosome 11 was the only chromosome consistently retained by the hybrids, the putative clock would have to reside on this chromosome (26). However, these results could not distinguish whether globin gene expression and switching in this system were due to a *cis*-active mechanism or involved *trans*-active control by other genes encoded on chromosome 11.

The human erythroblast × MEL cell hybrids provide a useful experimental system for analyzing the developmental regulation of human globin genes. However, two difficulties make this system less than optimal. Obtaining human fetal erythroid cells over a range of gestational ages is extremely difficult. Second, chromosome 11 does not contain a metabolically selectable marker; hybrid cells tend to lose human chromosomes quite rapidly, and therefore selection for the retention of chromosome 11 is necessary at weekly intervals on the basis of a cell surface antigen encoded by the chromosome (40). These problems might be overcome by using transgenic mouse cells as the source of the human β-globin gene cluster, since cells from early gestational ages are readily available. Hybrids formed by fusion of cells from the same species tend to retain both sets of chromosomes; therefore, hybrids selected for any metabolic marker are likely to contain the genes of interest, in this case the human globin transgenes.

We have demonstrated the suitability of transgenic mouse erythroblast × MEL cell hybrids as an experimental system for examining the control of human γ- and β-globin gene expression and its developmental regulation. We have shown, using mice transgenic for different globin gene constructs and of different gestational ages, that the initial pattern of human γ and β gene expression in these hybrid cells is determined by the pattern of expression in the donor cells at the time of fusion. It does not appear to be influenced by the *trans*-acting environment of the donor erythroblasts or of the MEL cells, suggesting that a major part of the regulatory process must involve stable *cis*-active modifications of the globin gene clusters. Switching from γ gene to β gene expression was observed on prolonged culture of hybrids derived from transgenic embryonic erythroblasts, with a time course similar to that observed in human fetal erythroblast hybrids. As switching in these hybrids showed no relationship to the time course in the transgenic mice, it is unlikely to be regulated by a developmental clock.

MATERIALS AND METHODS

Transgenic mice. TgN(HS2$^{G}\gamma^{A}\gamma^{-117}\delta\beta$)J14 and TgN(HS2$^{G}\gamma^{A}\gamma^{-117}\delta\beta$)J17 transgenic lines contain the LCR element 5′HS2 linked to a 39-kb fragment spanning the human $^{G}\gamma^{A}\gamma\delta$ and β genes in their normal genomic configuration (29). This construct contains a G-to-A substitution at position −117 in $^{A}\gamma$, a mutation which in vivo in humans confers the phenotype of hereditary persistence of fetal hemoglobin. LCRε$^{G}\gamma^{A}\gamma\delta\beta$ mice (line 72) contain a 70-kb fragment that was made by joining together two cosmids spanning the whole β-globin gene cluster (39).

Transgenic mice with a β-globin gene construct containing a neomycin resistance (*neo*) gene were also generated (19). A 1.1-kb *Xho*I-*Sal*I fragment was isolated from the HSVtk-neo plasmid (Stratagene) and inserted into the *Eco*RV site of the pN2γβ1 plasmid of Morley et al. (30). This places the *neo* gene at the 3′ end of this HS2$^{A}\gamma\beta$ construct, approximately 2 kb beyond the β-globin gene and in the same transcriptional orientation. The HS2$^{A}\gamma\beta$neo fragment was released by *Not*I digestion and used to generate three lines of transgenic mice. One of these lines, TgN(HS2$^{A}\gamma\beta$-neo)N4, was used in this study.

Cell lines. The MEL cells used were a semiadherent derivative of line 585, deficient for both adenosine and hypoxanthine phosphoribosyltransferases, and a semiadherent, thymidine kinase-negative derivative of line 707. Cells were maintained in RPMI 1640 (RPMI) with 15% fetal calf serum (FCS), glutamine, penicillin, and streptomycin and were harvested in log phase for fusion experiments.

Isolation of erythroid and nonerythroid cells. Embryonic and fetal cells were obtained from mice following the mating of homozygous transgenic mice with wild-type (C57BL/6 × CBA)F_1 females; the morning on which a copulatory plug was observed was considered to be 0.5 days postcoitus. Peripheral blood cells were collected by allowing the embryos and fetuses to bleed out into RPMI containing 10 U of preservative-free heparin per ml. The cells were then washed twice in RPMI to remove residual heparin prior to fusion.

Fetal livers were dissected out and mechanically disrupted in RPMI by gentle scraping with a scalpel blade. This released the more loosely attached erythroblasts from stromal material, which was removed by sedimentation.

Adult erythroid cells were obtained from the spleens of mice made anemic by injections of acetylphenylhydrazine (37). Gentle mechanical disruption of the spleen released a single-cell suspension which could be separated from tissue stroma and cell clumps by sedimentation.

An aliquot of each erythroid cell sample was removed before fusion to measure the in vivo globin gene expression.

Cell fusion. Polyethylene glycol (PEG) fusions were carried out on monolayers of cells in 25-cm^2 flasks by adapting the method for producing heterokaryons described by Baron and Maniatis (4). Approximately 1×10^7 to 2×10^7 blood, liver, or spleen cells were added in 3 ml of medium (RPMI, 15% FCS) to 25-cm^2 tissue culture flasks pretreated with poly-L-lysine (Sigma); the cells were allowed to adhere for 15 to 30 min. Approximately 2×10^7 to 3×10^7 MEL cells were added to the primary cell layers and allowed to adhere for 20 to 30 min to give a mixed monolayer. The medium containing excess cells was discarded, and the monolayer of cells was rinsed three times in serum-free RPMI. Two milliliters of 50% PEG (molecular weight, 1,300 to 1,600; Sigma) in RPMI, prewarmed to 37°C, was added to the cells for 1 min and then diluted with 10 ml of RPMI added slowly, with gentle mixing, over a period of ~2 min. The diluted PEG was removed, and the cells were washed twice with RPMI and incubated in medium with FCS. After 2 h, the fused cells were detached from the flask by gentle pipetting or, if strongly adherent, by scraping with a rubber policeman. The detached cells were distributed into 5 or 10 pools in uncoated flasks. The MEL cells and the majority of the fused cells reattached to the surface, while unfused primary cells and cells killed by the fusion process remained in suspension. After 24 h, the medium and cells in suspension were discarded and replaced with selective medium (RPMI, 15% FCS, 10^{-5} mol of methotrexate per liter, 10^{-4} mol of adenine per liter, 3×10^{-5} mol of thymidine per liter, 10^{-4} mol of hypoxanthine per liter). The selective medium was renewed after ~3 days to remove the debris of detached dead cells, and at this stage small hybrid colonies could be detected growing on the bottom of the flask. These colonies were counted 5 to 7 days after fusion.

For production of irradiation hybrids, cells were collected in medium and then maintained on ice during irradiation (50 Gy at a dose rate of 2.5 Gy/min) with a cesium γ-ray source. Fusion was carried out as described above except that the selective medium consisted of RPMI–15% FCS containing 0.8 mg of Geneticin (Gibco) per ml.

Hybrid cells were usually maintained in selective medium. After 2 to 3 weeks, cell numbers had increased to 1×10^7 to 2×10^7 cells per flask; cells were harvested for cryostorage, DNA analysis, and induction of erythroid differentiation. Cells were induced in log phase by adding 5 mmol of hexamethylene bisacetamide per liter or 3 mmol of hexamethylene bisacetamide per liter plus 25 μmol of hemin per liter and were harvested for RNA analysis after 48 to 72 h.

Karyotyping. Hybrid cells were incubated with 0.2 μg of colcemid per ml for 2 h, and stained karyotypes were obtained by standard methods (7). Twenty to fifty random metaphases were photographed for chromosome counting.

RNA analysis. RNA was prepared by the method of Chomczynski and Sacchi (10) and was analyzed by the quantitative RNase protection assay of Zinn et al. (44). The following RNA probes were used for detecting mouse transcripts: mouse α, pSPMαS (R. W. Jones, University of Oxford); mouse β major, pSP64Mβ134 (3); and mouse ε, pSP64Mε (3). Probes for the human transcripts were as follows: human β, pGβ1 (29); human γ, pGγ2 (29); and human $^{G}\gamma/^{A}\gamma$, pBA$^{A}\gamma$ (17).

Hybrid cell RNA (1 to 10 μg) or transgenic mouse RNA (0.1 to 1 μg) was hybridized with up to four different [α-^{32}P]GTP-labelled probes, always including

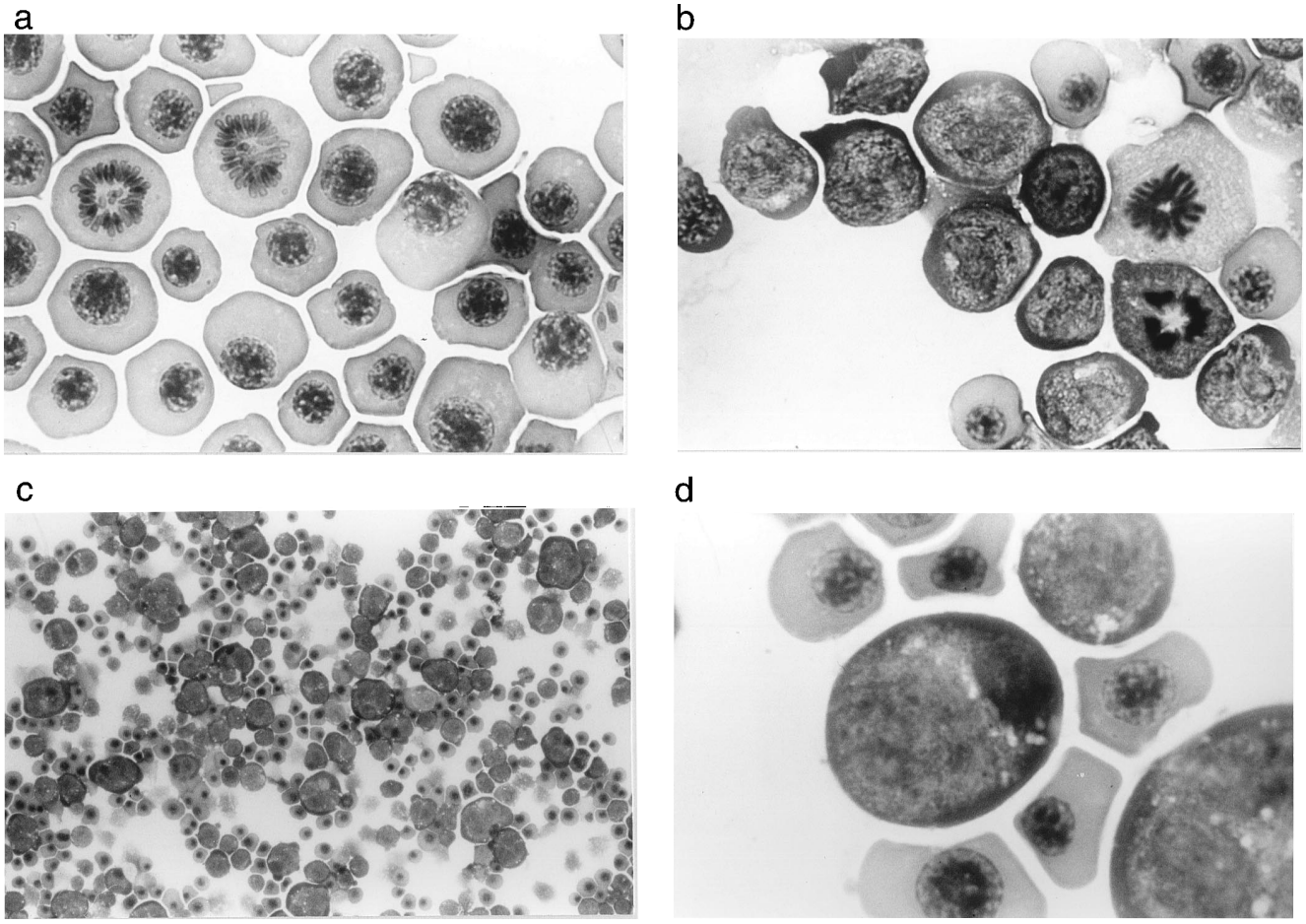


FIG. 1. (a) Peripheral blood cells from an 11.5-day mouse embryo. (b) Fetal liver cells at 11.5 days of gestation. (c) The appearance of MEL × 11.5-day blood cells, 2 h after fusion, showing a high proportion of polykaryons. (d) A dikaryon with nuclei of different morphologies, likely to be a heterokaryon.

the mouse α probe as an internal control. Quantitation of the RNase-protected fragments was performed by excising the bands from the gel and counting them in scintillation liquid. For accurate quantitation of RNAs present in low proportions, a 10- to 20-fold larger amount of RNA was hybridized separately with the probe for the less abundant RNA than was hybridized with the probe for the more abundant RNA. The two hybridization solutions were then mixed immediately before RNase treatment.

RESULTS

Cell purity and frequency of hybrid formation. For each fusion experiment, cytospin preparations of the cell populations obtained from the transgenic mouse tissues were examined to assess the purity of the cell types obtained (Fig. 1). The results are summarized in Table 1, which also shows the rates of hybrid generation from the various tissues at different stages of development. The peripheral blood cells of 11.5- to 13.5-day embryos consisted almost entirely of nucleated yolk sac erythroid cells which gave a relatively low rate of fusion with MEL cells. At 11.5 days of gestation, erythropoiesis has started in the fetal liver; the majority of cells obtained were basophilic erythroblasts, although at this stage there were significant numbers of circulating yolk sac cells. As erythropoiesis increased in the fetal liver, all maturation stages of the erythroid series were found and the proportion of contaminating yolk sac cells declined. Early fetal liver cells consistently gave the highest rate of fusion, 10- to 50-fold greater than that of yolk sac cells, suggesting that even at 11.5 days of gestation, any contaminating yolk sac cells were unlikely to contribute significantly to the fetal liver hybrids produced.

Hybrid cell growth and inducibility. The majority of cell fusions were carried out with the 585-A MEL cell line, which lacks both adenosine and hypoxanthine phosphoribosyltransferases. However, to demonstrate that the results were not

TABLE 1. Purity of the cell populations used for fusion and the rate of hybrid cell formation

Source of cells	% of total nucleated cells			No. of colonies/10^6 cells fused
	Yolk sac erythroblasts	Definitive erythroblasts	Non-erythroid cells	
Embryonic blood				
11.5 days	>99	0	<1	0.5–1.0
12.5 days	>99	0	<1	0.5–1.0
13.5 days	98	<1	<2	0.5–1.0
Fetal liver				
11.5 days	20–50	50–70	5–10	20–50
12.5 days	10–30	50–80	5–10	5–20
13.5 days	5–15	65–85	5–10	5–10
15.5 days	<2	75–90	10–20	3–10
Phenylhydrazine-treated adult spleen	0	65–80	20–35	0.5–1

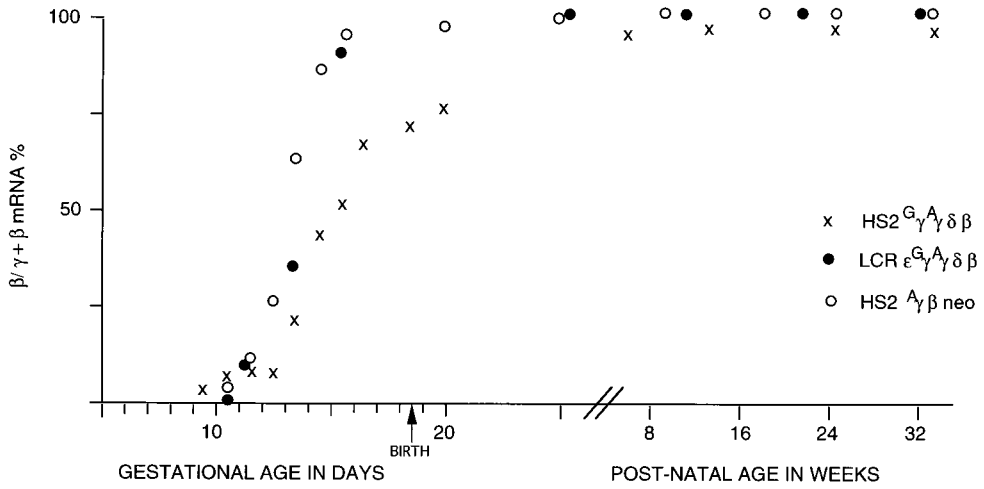


FIG. 2. Pattern of peripheral blood γ-to-β gene switching in vivo for each of the three transgenic mouse lines. Note that in the LCRεγγδβ line there is also expression of the human ε gene from 8.5 to 13.5 days of gestation. The data were derived from RNase protection assays of 3 to 10 individual embryos or adults at each time point.

peculiar to this line, some fusions were carried out in parallel with the 707-A MEL cell line, lacking thymidine kinase. Fusion rates were similar with both MEL cell lines, and no differences in growth, inducibility, or subsequent globin gene expression in the hybrids produced by the two lines were observed.

Developing hybrid cells grew as adherent colonies on the bottom of the flasks, with a morphological phenotype similar to that of the MEL cells. In general, they grew at approximately similar rates, such that the colonies were clearly detectable 5 to 7 days after cell fusion, at which time they were counted to give an estimate of the number of clones contributing to each hybrid cell pool. Small, slowly growing hybrids would have been missed at this stage, as would nonadherent colonies. It is likely, however, that the more rapidly emerging colonies would contribute proportionately more cells to a pool, and therefore this is a more reliable time to count colonies than later, when secondary colonies were forming. Individual clones either were picked at ~8 to 10 days after fusion, selecting only well-separated colonies from flasks with low colony numbers, or were picked from semisolid methylcellulose cultures.

Sufficient cells for RNA and DNA analysis, $\sim 1 \times 10^7$ to 2×10^7, were normally available 2 to 3 weeks after erythroid cell fusion, depending on the number of hybrid clones per flask. This gives a population doubling time of less than 24 h; given that there is some cell death if cells lose the chromosome with the selectable marker, this finding suggests that the cell division time of the hybrids cannot be much longer than the 16 h of the parental MEL cells.

Erythroid maturation of the hybrid cells was induced with 5 mM hexamethylene bisacetamide. The degree of induction was monitored at the time of cell harvesting for RNA analysis (72 h) by the redness of the cell pellet. All the hybrid cell populations showed induction to some degree; the majority produced pellets as red as induced MEL cells and contained high levels of mouse α and β^{maj} mRNA (data not shown).

Human γ- and β-globin gene expression in the hybrid cells. Somatic cell hybrids were made from two lines of transgenic mice; J14 contains ~2 copies of the $HS2^{G}\gamma^{A}\gamma^{-117}\delta\beta$ globin gene construct (29), while line 72 carries a single copy of a 70-kb $LCR\varepsilon^{G}\gamma^{A}\gamma\delta\beta$ construct containing the whole of the β-globin gene cluster, including the LCR (39). Both lines show developmental regulation of the human genes in vivo. However, while the switch from γ to β gene expression begins at the same time (~10.5 to 11.5 days of gestation) in both lines, it is completed in the $LCR\varepsilon^{G}\gamma^{A}\gamma\delta\beta$ mice by ~16.5 days of gestation but not until after birth in the $HS2^{G}\gamma^{A}\gamma^{-117}\delta\beta$ lines (Fig. 2).

Expression of the human γ and β genes in MEL × transgenic erythroid cell hybrids on initial induction was readily detectable by RNase protection assay, with human globin RNA levels of up to 70% of mouse globin RNA levels. Representative autoradiographs are shown in Fig. 3A, and the results are summarized in Fig. 3B. The γ/γ+β mRNA ratios in 11.5- to 13.5-day embryonic blood erythroblasts in vivo range from 75 to 100%, as shown by the vertical lines in Fig. 3B. Hybrids derived from these cells show predominantly the same pattern, with 17 of 47 hybrid pools producing exclusively γ mRNA and 39 of 47 having greater than 75% γ mRNA production. Similar results were obtained with individual hybrid clones, and there was no significant difference between hybrids produced from the two different transgenic lines.

Hybrids produced from adult erythroblasts showed predominantly β gene expression. In $LCR\varepsilon^{G}\gamma^{A}\gamma\delta\beta$ adult erythroid cells, the γ genes are almost completely repressed in vivo, and γ mRNA was largely undetectable in hybrids formed from these cells. In the $HS2^{G}\gamma^{A}\gamma^{-117}\delta\beta$ mice, up to 10% γ mRNA is found in adults, heterogeneously distributed among the erythrocytes (29). More variable γ gene expression was observed in hybrids generated from these cells, although again β mRNA predominated and nearly half of the hybrid pools and clones contained exclusively β mRNA.

Hybrids formed from fetal liver erythroblasts produced a wide array of γ and β gene expression ratios. In the $HS2^{G}\gamma^{A}\gamma^{-117}\delta\beta$ lines, no obvious differences in this range were observed in hybrids from fetal liver cells collected between 11.5 and 15.5 days of gestation, during which time γ gene expression declined from ~85% to ~50% in vivo. In $LCR\varepsilon^{G}\gamma^{A}\gamma\delta\beta$ hybrids, a range of values was observed only in those from 11.5-day fetal liver cells. Expression was almost exclusively β in fetal liver hybrids from 13.5 or 15.5 days of gestation, reflecting the fact that the switch is almost complete in this organ at these stages.

RNase protection analysis using a probe from the 3′ region of the γ gene, which can distinguish $^{G}\gamma$ and $^{A}\gamma$ transcripts, dem-

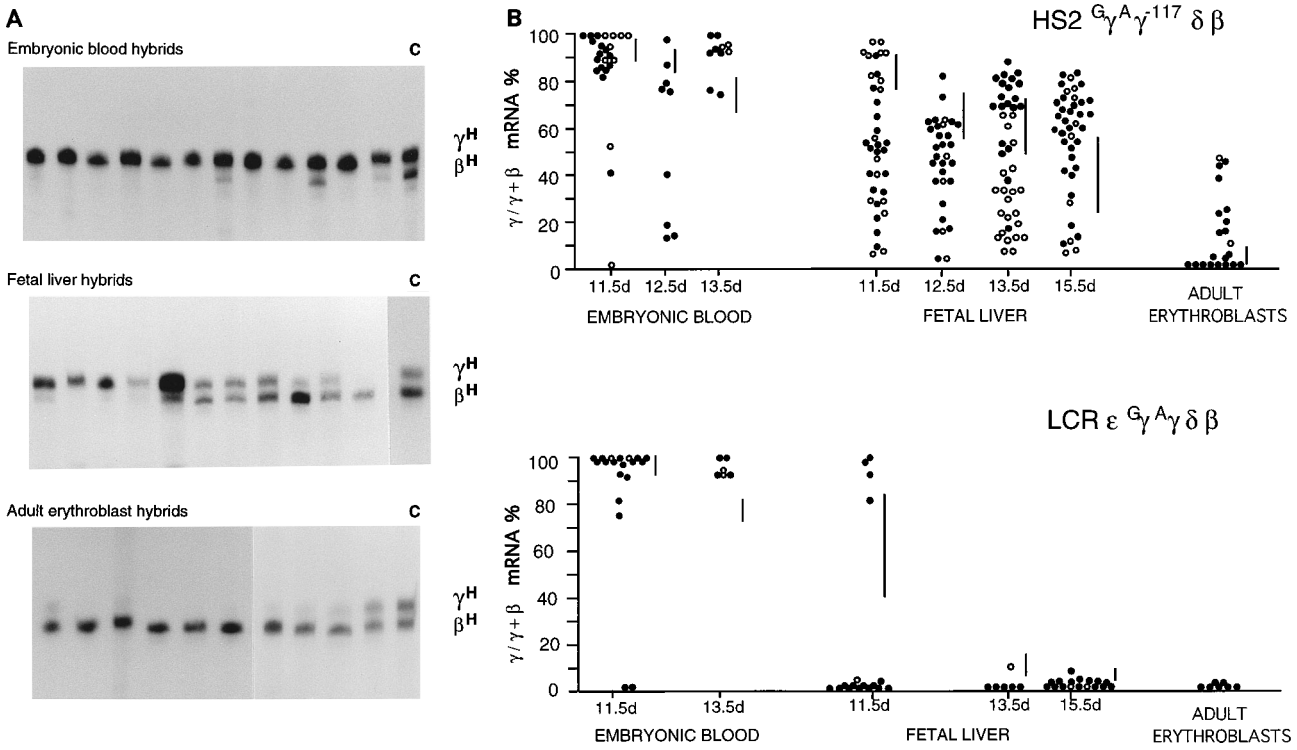


FIG. 3. (A) RNase protection assays to demonstrate the proportions of human γ and β mRNAs in HS2$^{G}\gamma^{A}\gamma^{-117}\delta\beta$ hybrids generated from 11.5-day embryonal blood (top), 11.5-day fetal liver (middle), and adult spleen erythroblasts (bottom). C, control mixture of normal adult and cord blood RNA. Each lane represents hybridization of 5 to 10 μg of RNA from an individual hybrid pool or clone. (B) The proportion of γ to β mRNA on the initial induction of HS2$^{G}\gamma^{A}\gamma^{-117}\delta\beta$ and LCR$\varepsilon^{G}\gamma^{A}\gamma\delta\beta$ hybrids from the sources indicated. Closed symbols represent hybrid pools; open symbols represent individual clones. The vertical lines show the ranges of γ and γ+β mRNA levels in vivo in the samples used for the fusions. The suffix "d" indicates days.

onstrated that both genes were expressed in HS2$^{G}\gamma^{A}\gamma^{-117}\delta\beta$ hybrids from all stages of development (data not shown).

These results show that the pattern of human globin gene expression in the hybrids closely matched the pattern of expression in the transgenic erythroblasts at the time of fusion, even to the degree that differences in the timing of switching between the two different transgene constructs and the difference in γ gene expression in adult life between the two lines were recapitulated in the hybrids. To the extent that most of the embryonic blood hybrids produced exclusively or predominantly γ mRNA, it would appear that in the mouse × mouse hybrid system, the adult *trans*-acting environment of the MEL cell does not play a major role in determining gene expression. However, these results do not allow us to determine whether the continued γ gene expression in these mouse × mouse hybrids was due to *cis*- or *trans*-acting mechanisms, since the genes responsible for any putative embryonic *trans*-acting factors in the donor embryonic cells could still remain active in the hybrid cells.

Globin gene expression in irradiation hybrids. Irradiation hybrids were therefore used to determine whether *trans*-acting factors expressed from donor cell chromosomes determine transcription in the hybrids. Irradiation levels of 50 Gy break chromosomes into fragments of less than 2 to 3 Mb, and after fusion of irradiated cells, hybrids retain only a small number of donor fragments rather than an intact donor genome (36, 42). Such hybrids require a selectable marker linked to the transgenes, necessitating the use of a smaller globin gene construct. Therefore, three transgenic mice lines containing the HS2$^{A}\gamma\beta$ *neo* fragment were generated. The in vivo switching pattern of this construct was similar in all three lines to that observed in the LCR$\varepsilon^{G}\gamma^{A}\gamma\delta\beta$ mice; there was ~100% γ gene expression at 8.5 to 10.5 days of gestation, switching rapidly to ~100% β gene expression by day 14.5 (Fig. 2).

Embryonic blood samples were pooled from one litter, and half of the sample was irradiated. Both irradiated and unirradiated cells were separately fused with MEL cells. The irradiation hybrid pools were karyotyped, and chromosome numbers compared with those in nonirradiation hybrids. The distribution of chromosome numbers in the nonirradiated HS2$^{A}\gamma\beta$neo hybrids ranged from pseudodiploid to pseudotetraploid, and the range was indistinguishable from those obtained with LCR$\varepsilon^{G}\gamma^{A}\gamma\delta\beta$ and HS2$^{G}\gamma^{A}\gamma^{-117}\delta\beta$ hybrids (Fig. 4A). The chromosome number in all irradiation hybrids was pseudodiploid, the median number of 39 being the same as that of the MEL cell line (Fig. 4B). There was no evidence of unusually long chromosomes. This finding demonstrates that only limited amounts of erythroblast DNA had been transferred to the MEL cells from the irradiated erythroid cells and therefore that the only material consistently present in the hybrids would be that surrounding the selectable *neo* gene.

The globin gene expression patterns of irradiation and nonirradiation hybrid pools on initial induction are shown in Fig. 4C and clearly demonstrate no significant difference between the two groups. All hybrids continue to produce γ mRNA to a variable degree. This lack of difference in gene expression between hybrids that contain most of the donor cell genome and those with only a limited amount of donor cell DNA (which will be variable from clone to clone) strongly suggests that the continued γ gene expression in these hybrids is not due

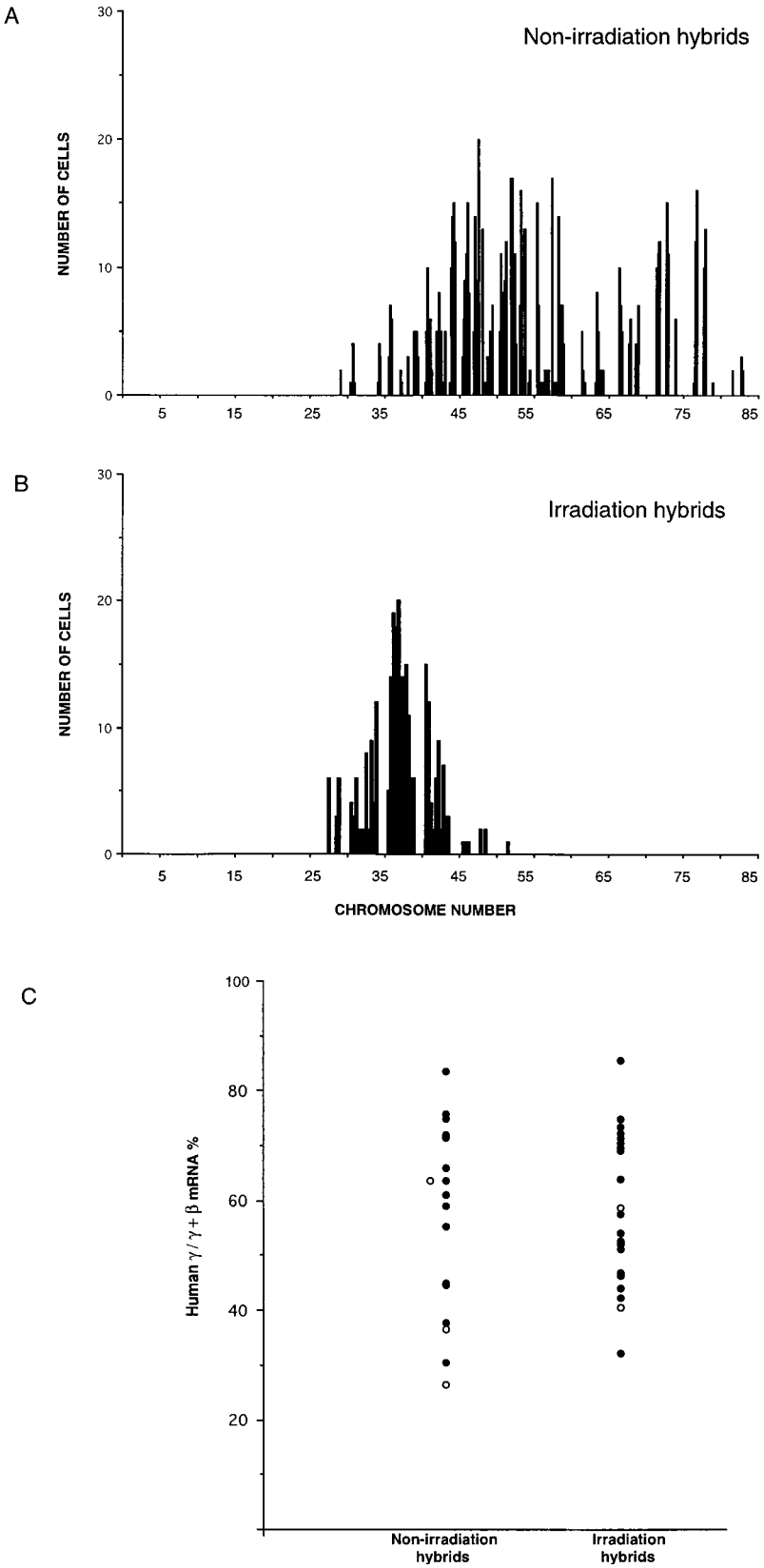


FIG. 4. (A) Chromosome number in ~30 cells from each of 25 individual unirradiated hybrid pools and clones representing $HS2^{G}\gamma^{A}\gamma^{-117}\delta\beta$, $LCR\varepsilon^{G}\gamma^{A}\gamma\delta\beta$, and $HS2^{A}\gamma\beta$neo lines. No difference in the range of chromosome numbers was observed between the various lines. (B) Chromosome number in ~25 cells from each of 12 individual hybrid pools or clones from $HS2^{A}\gamma\beta$neo cells following irradiation. (C) Comparison of the γ/β mRNA ratios in irradiation and nonirradiation hybrid pools (●) or clones (○) bearing the $HS2^{A}\gamma\beta$neo transgene.

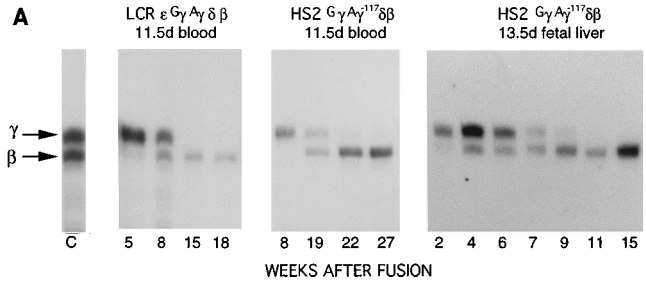


FIG. 5. (A) RNase protection assays to demonstrate in vitro switching from γ to β mRNA with increasing time in culture in three representative hybrid clones. C, control mixture of adult and cord blood RNA. (B) Pattern of in vitro globin gene switching with increasing time in culture in $HS2^{G}\gamma^{A}\gamma^{-117}\delta\beta$ hybrid clones. The suffix "d" indicates days.

to continued production of embryonic *trans*-acting factors derived from the donor cells.

Globin gene switching in vitro. In the human fetal erythroblast × MEL cell hybrid system, it was demonstrated that prolonged culture of the hybrids resulted in a switch from γ to β mRNA production in vitro over a period of 20 to 50 weeks (32). We therefore determined whether in vitro hemoglobin switching would occur in the transgenic mouse × MEL cell system and, if so, with what time course.

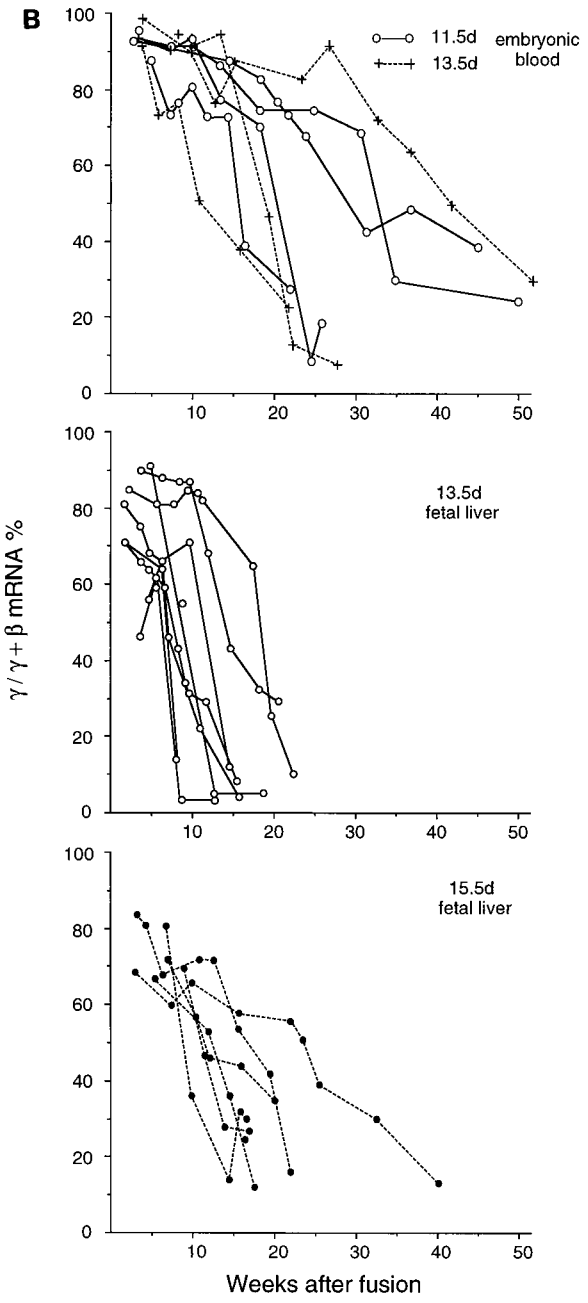
Cells from hybrid pools which expressed predominantly or exclusively γ mRNA on initial induction were cloned in semisolid medium and maintained in culture for periods of up to 1 year. Samples were removed for erythroid induction and RNA analysis at 2- to 4-week intervals. The longitudinal patterns of γ and β mRNA levels in representative clones are shown in Fig. 5A, and the results for the $HS2^{G}\gamma^{A}\gamma^{-117}\delta\beta$ hybrids are summarized in Fig. 5B. Nearly all of the hybrids show a decline in the γ/γ+β mRNA ratio with increasing time in culture, with many showing a more or less complete switch from γ to β mRNA expression. The time span taken to complete switching ranged from ~10 weeks to greater than 40 weeks after fusion. Similar results were also obtained with the $LCR\varepsilon^{G}\gamma^{A}\gamma\delta\beta$ hybrids (Fig. 5A).

The data in Fig. 5 include hybrids generated from both blood and fetal liver cells taken between 11.5 and 15.5 days of gestation. There is no clear-cut correlation between any of these variables and the time taken to switch in vitro; the apparent later switching in some of the embryonic blood hybrids may reflect higher initial levels of γ gene expression rather than a tissue-specific difference.

DISCUSSION

Developmental regulation of globin gene expression is likely to involve a hierarchy of controls at both the cellular and molecular levels. *trans*-acting factors interacting with the globin genes at the time of transcription could be the major determinants of gene expression; in this case, qualitative or quantitative changes in these factors must occur at different stages of development. However, it is equally possible that interactions with *trans*-acting factors at some earlier stage in development or differentiation establish the subsequent differential accessibility of the genes to transcription factors (43). The results presented here provide direct evidence that the latter mechanism may be a major regulatory feature of the control of fetal versus adult globin gene expression.

The pattern of human γ and β gene expression in somatic cell hybrids between transgenic mouse erythroblasts of different developmental stages and MEL cells reproduced the pattern of expression of the donor cells in vivo. Thus, hybrids formed from embryonic blood cells continued to express predominantly their γ genes. Adult erythroblast hybrids transcribed largely their β globin genes, with much tighter regulation in the $LCR\varepsilon^{G}\gamma^{A}\gamma\delta\beta$ hybrids than the $HS2^{G}\gamma^{A}\gamma^{-117}\delta\beta$ hybrids. This may well reflect the difference in vivo, as γ gene expression is almost completely repressed in adult mice with the former construct but continues at the 5 to 10% level with the latter one. Since there is a heterocellular distribution of γ chains in these animals (29), those cells with relatively high γ gene expression may well have produced hybrids that continue to express this gene. Fetal liver hybrids generated from $HS2^{G}\gamma^{A}\gamma^{-117}\delta\beta$ cells expressed both γ and β genes to a variable degree, whereas hybrids made from $LCR\varepsilon\gamma\gamma\delta\beta$ cells,



which switch more rapidly in vivo, again showed much tighter regulation of these genes.

This pattern of globin gene expression in hybrids could reflect a number of influences, including *trans*-acting factors present in the MEL cells, *trans*-acting factors carried over from the incoming donor cell, or the perpetuation of *cis*-active modifications to the human globin transgene. If the *trans*-acting factor complement of the MEL cells were the major determinant of globin gene expression in this system, we would have expected to see expression of the human β gene in embryonic cell hybrids. Both lines of MEL cells used in these experiments transcribe only their adult globin genes, and high levels of mouse α and β^{maj} mRNAs continued to be produced in all the hybrids. However, no human β gene expression was seen in the majority of the embryonic blood cell hybrids, strongly suggesting that this gene is unavailable to interact with the adult *trans*-acting factors of the MEL cells, even after multiple rounds of cell division and in a transcriptionally active environment. The inactive conformation of this gene on the incoming chromosome must therefore be extremely stable.

The possibility that continued expression of embryonic *trans*-acting factors from the donor cell chromosomes is responsible for the continued production of human γ mRNA in embryonic cell hybrids is made extremely unlikely by the results from the irradiation hybrids. When the donor cells were irradiated prior to fusion so that only limited amounts of their genetic material were transferred to the hybrids, there was no difference in their pattern of expression compared with that of hybrids formed from nonirradiated cells containing the same construct. Furthermore, the observation that in the γ gene-expressing hybrids there is no continued expression of the endogenous or transgenic embryonic ε, βh1, or ζ gene (38a) strongly supports the contention that continued embryonic *trans*-acting factor production is not necessary for γ gene expression in this system. In these embryonic cell hybrids, the γ globin genes must be in an extremely stable, active conformation that is maintained after cell fusion despite the altered *trans*-acting environment.

These results demonstrate, therefore, that neither the *trans*-acting factors of the MEL cells nor those of the donor cells appear to play a major role in determining human γ and β gene expression in the hybrids. Rather, the conformation of the complex at the time of fusion, which is stable and heritable through both replication and transcription, determines gene expression. The results presented here extend those obtained previously for hybrids between human fetal liver erythroblasts and MEL cells. The pattern of expression in those hybrids also mirrored that in vivo and was solely dependent on the presence of chromosome 11, which carries the human β globin gene cluster (26, 32). In those experiments, it was not possible to distinguish between a *cis*-active modification of the globin gene cluster or the expression of a *trans*-acting factor from that chromosome. Our demonstration that fragmented chromosomes behave similarly to intact chromosomes makes the former model much more likely.

Globin gene expression in the hybrid cells contrasts markedly with the results obtained when the same or similar constructs were stably transfected into MEL cells. When constructs containing both γ and β genes were introduced into MEL cells by electroporation, there was no developmentally regulated pattern of expression; $^{G}\gamma$, $^{A}\gamma$, and β genes were all expressed in approximately equal amounts (30). This result provides further evidence that the *trans*-acting factor complement of erythroblasts is not sufficient for selective gene expression. The contrast between the regulated expression of these genes in erythroid cell hybrids and the unregulated expression

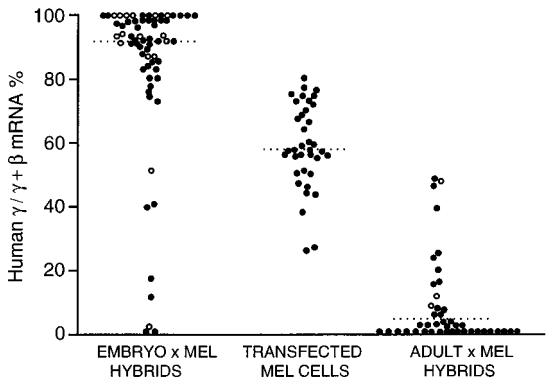


FIG. 6. The ratio of γ to β gene expression in embryonic blood × MEL and adult erythroblast × MEL hybrid pools (●) or clones (○) (combination of data for both transgenic lines shown in Fig. 3) compared with the expression of $HS2^{G}\gamma^{A}\gamma^{-117}\delta\beta$ and $HS2^{A}\gamma\beta$ fragments transfected into MEL cells (data from reference 30).

in transfected MEL cells is shown in Fig. 6. While embryonic cell hybrids have a mean γ/γ+β mRNA ratio of 84% and adult erythroblast hybrids have a ratio of 9%, the value in transfected cells is 58%. Thus when the same constructs were introduced as naked DNA into MEL cells, they behaved quite differently from when they were introduced as intact chromatin which had been through a normal developmental history. It may be of relevance that similar results have been observed for the human keratin 18 gene (1, 31).

On the basis of these clear differences between the results with mouse or human × MEL cell hybrids on the one hand and transfected MEL cells on the other, we suggest that the chromatin structure of the globin gene complex is an important determinant of the expression of these genes. Given the similarity in the patterns of expression in vivo and in the hybrids, it seems most likely that this structure is established during development of the erythroblasts themselves, prior to cell fusion. In both transgenic mouse embryonic cells and human fetal erythroblasts, the conformation of the cluster would ensure that the γ genes but not the β genes are available for transcription. After transfer to MEL cells by cell fusion, this conformation must be maintained (at least during the initial cell divisions) despite the fact that the machinery for transcribing the endogenous mouse adult globin genes is intact and active. In adult erythroid cells, this situation with the γ and β genes is reversed, with the β gene established in the appropriate structure for expression, a conformation that is maintained in the hybrids. We believe that the MEL cells, therefore, are capable of perpetuating regulatory information transferred with the intact chromosome but appear to be unable to reprogram this stably modified conformation or to establish such a conformation on transfected DNA. This raises the possibility that the critical chromatin conformation may be imparted to the β-globin locus at an earlier stage in erythroid cell maturation than in the MEL cell, which is generally considered to be equivalent to the CFU-E, a late progenitor cell. The nature of the epigenetic changes which bring about the stable, stage-specific conformations are presently unknown but presumably involve modifications to the chromatin structure of the gene cluster; they may include the methylation pattern of the cluster, positioning of nucleosomes, DNA supercoiling, intranuclear compartmentalization, or nuclear matrix attachment.

Data from heterokaryon experiments, in which gene expression is examined shortly after cell fusion and before nuclear fusion, have shown that nonexpressed globin genes can be

activated by *trans*-acting factors, apparently in a developmental stage-specific manner (3, 4). In heterokaryons between MEL cells and a wide range of human nonerythroid cells, expression of the human β gene is regularly observed. Why, then, do we observe little or no β gene expression in the 11.5-day embryonic cell hybrids? There are a number of differences between the two systems; heterokaryons are transient expression systems in which initial events may not be maintained after replication and the reestablishment of original conformations may occur in stable hybrids. Most of the heterokaryon experiments involved fusions of two immortalized cell lines, while our hybrid system uses primary erythroid cells as one of the fusion partners. In the heterokaryons, the levels of induced globin gene expression are very low and the proportion of cells which respond is unknown. Similarly, the ratio of nuclei from each cell type present in the heterokaryon is unknown, and induction could conceivably result only when there is a high ratio of inducing to responding nuclei. Reconcilement of the data from the two systems may need to await better knowledge of the *cis* and *trans* factors involved. However, the combination of the transfection and hybrid data on one hand with data from heterokaryons and *trans*-acting factors (8, 20, 22, 25) on the other hand suggests that both epigenetic processes and *trans*-acting factors contribute to the developmental regulation of globin genes.

With prolonged culture, the mouse × mouse hybrid cells which initially expressed the human γ genes switched to largely β mRNA production. The time course of this switching is very similar to the range observed with human fetal erythroblast × MEL cell hybrids (32, 45). The timing of in vitro switching in the human hybrids was within the period when these cells would have switched in vivo, and evidence was presented that the time of switching in the hybrids was correlated with the gestational age of the donor cells at the time of fusion. This finding led to the suggestion that the process was controlled by a developmental clock encoded on chromosome 11. Clearly there is no temporal relationship between in vivo switching in the transgenic mice and in vitro switching in the mouse hybrid system. Indeed, if γ-to-β gene switching in the mice were dictated by a developmental clock, one would have expected it to have been completed in the hybrid cells between the time of fusion and first analysis. Therefore, since one might expect the same mechanism to be responsible for in vitro switching in both hybrid systems, the data presented here raise questions as to whether the timing of switching in the human hybrids is related to gestational age fortuitously rather than causally.

One interpretation of the switching process in the transgenic mouse × MEL cell hybrids is that the epigenetic structural information carried by the chromosome in the hybrid cells is ultimately lost with continuing time in culture. This loss may occur stochastically, giving rise to the wide variability of switching times from one hybrid to another. Once the stable conformation which promotes γ gene expression is lost, expression of the β globin gene is allowed, presumably as a result of a new stable conformation since the cells do not show the equal γ and β expression pattern of transfected MEL cells, nor does the γ gene appear to be reexpressed once it has been switched off.

The results presented here clearly demonstrate that *trans*-acting factors present in late erythroid cells cannot be the sole determinants of γ versus β gene expression; rather, they suggest that epigenetic modifications acquired during development and differentiation may play a major role in determining expression of these genes in the hybrid cells. The somatic cell hybrid system described here should be of general value in elucidating the relative importance of *cis* and *trans* events in developmental gene regulation in other systems for which appropriate transgenic mice and immortalized cell lines are available.

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