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Tissue-specific DNase I hypersensitive sites in a foreign globin gene in transgenic mice

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ABSTRACT

We have investigated the DNase I hypersensitivity of a hybrid mouse/human beta-globin gene in erythroid and non-erythroid cells of transgenic mice, to examine the relationship between the chromatin structure and the expression of an exogenous gene. The hybrid globin gene was previously shown to be expressed specifically in erythroid cells in some transgenic lines. The maximal level of hybrid globin mRNA accumulation was a few percent of the endogenous level, and we show here that this results from a low rate of transcription. In erythroid cells from two transgenic lines in which the hybrid gene is expressed, we detect a set of DNase I hypersensitive sites whose locations are indistinguishable from those in endogenous beta-globin genes. The hybrid globin gene contains no DNase I hypersensitive sites in transgenic mouse brain cells. Thus, the tissue-specific expression of the exogenous globin gene is reflected in, and perhaps mediated by, tissue-specific changes in chromatin structure.

INTRODUCTION

The transcriptional activation of a gene in a specific cell lineage during development appears to involve several steps, some of which are evident as detectable alterations in chromatin structure. These processes have been well studied in the case of the alpha and beta-globin genes of vertebrates. The activation of the globin genes during the differentiation of erythroid cells seems to include a complex series of chromatin changes, affecting different regions within and flanking the genes (1-11). One type of event that can be observed relatively easily is the appearance of specific sites that are highly sensitive to cleavage by nucleases or other chemical agents, presumably reflecting the binding of non-histone proteins and the consequent modification or displacement of nucleosomes (1-7,9,11-14). It has been suggested that the formation of these nuclease-hypersensitive

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regions represents a commitment to express a gene rather than simply a consequence of transcription, because their formation can be divorced from actual transcription under a number of experimental conditions (15,16).

Cloned beta-globin genes, introduced into the mouse germ line by microinjection into the zygote, are in most cases expressed specifically in erythroid cells (17-19) at an appropriate developmental stage (18-21), despite their insertion into diverse chromosomal locations (22). During the development of a transgenic mouse, such a gene is potentially exposed to all the trans-acting signals involved in the activation of the endogenous beta-globin gene in erythroid cells and its inactivation in other cell types. Therefore, it is possible that the exogenous and endogenous genes are regulated through a similar or even identical series of events, entailing the same alterations in chromatin structure. However, there are several reasons to suspect that tissue-specific expression in a transgenic animal might be achieved by only a subset of the normal regulatory events. First, it has been argued that cloned beta-globin genes introduced into cultured cells by transfection bypass some of the earlier regulatory events and immediately adopt an "open" chromatin configuration, in which they require only the presence of a positive activator to be transcribed (23-26). Globin genes in transgenic mice might be regulated through a similarly abbreviated mechanism. Second, the level of expression of an exogenous globin gene in different transgenic mice varies by several orders of magnitude, apparently as a function of chromosomal position, and the average level (per gene copy) is much lower than that of the endogenous gene (17-21). This suggests that some step in the normal activation of the beta-globin gene may fail to occur when the gene is inserted at abnormal chromosomal positions.

To investigate these issues, we have examined the DNase I hypersensitivity of an exogenous beta-globin gene in both erythroid and non-erythroid cells of several transgenic mouse lines. A preliminary account of some of our results has been published (18).

MATERIALS AND METHODS

Homozygous adult transgenic mice from lines 46 and 47 (ref. 17) were injected intraperitoneally on four consecutive days with 0.1ml of 0.9% phenylhydrazine, and were sacrificed on the sixth day. The spleens were removed and gently homogenized in anticoagulation buffer (2% acid citrate dextrose, 14% citrate phosphate dextrose, 84% phosphate buffered saline (PBS), 1mM PMSF; ref 27) in a teflon-glass homogenizer. Nucleated cells were isolated from the homogenate by centrifugation over Lymphocyte Separation Medium (Bionetics), and nuclei were prepared from cells banding at the interface, according to the procedure of Larsen and Weintraub (28). Brains were also removed, homogenized in PBS, and nuclei were similarly isolated from total brain cells. Livers and brains were dissected from homozygous transgenic fetuses of line 77 (ref. 17) on day 15 of gestation, and nuclei were prepared as described above. DNase I digestions were performed according to Sheffery et al. (7), using DNase I concentrations ranging from 2 - 16 ug/ml.

Nuclear runoff transcription experiments were performed as described (6), except that unincorporated nucleotides were removed by passing labelled, purified nuclear RNA over a Sephadex G-100 column. Nuclear runoff experiments were performed using nuclei from homozygous line 77 fetal livers on days 14 or 15 of gestation.

RESULTS

Relative rates of transcription of foreign and endogenous beta-globin genes in transgenic mice. We have previously described a series of transgenic mouse lines carrying a hybrid 5' mouse/human 3' beta-globin gene (29), several of which expressed the gene specifically in erythroid tissues (17). In these animals, which were hemizygous for the foreign gene, the steady state levels of hybrid mouse/human globin mRNA were no greater than 1-2% of the endogenous beta-globin mRNA level (17,20), suggesting a low rate of transcription. However, it remained possible that the gene might be transcribed at a normal rate, but that the hybrid

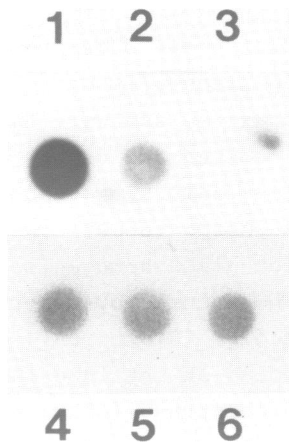

Figure 1. Nuclear runoff analysis of the relative rates of transcription of the foreign and endogenous beta-globin genes in transgenic line 77. 5ug of DNA from recombinant clones containing the mouse beta-major globin large intron (380 nt HindIII - PstI fragment; dot 1), the human beta-globin large intron (920 nt BamHI - EcoRI fragment; dot 2) and plasmid pBR322 (dot 3) were immobilized on nitrocellulose (34). ^{32}P-labelled nuclear runoff transcripts were isolated from fetal liver nuclei, and hybridized to the filter. The amount of RNA hybridized was measured by autoradiography and densitometry. The labelled RNA was then removed and the filter was rehybridized with labelled pBR322 DNA, to show that equal amount of each DNA were bound to the filter and accessible for hybridization (dots 4-6).

mouse/human globin mRNA might be inefficiently processed, or unstable.

To more directly compare the rates of transcription of the foreign and endogenous beta-globin genes, we performed nuclear runoff assays using nuclei from fetal liver cells (from transgenic line 77; refs. 17 and 20) and probes specific for the foreign and endogenous beta-globin genes. This procedure measures the relative number of RNA polymerase molecules transcribing specific genes, and thus their relative rates of transcription (30). As shown in Figure 1, the hybrid beta-globin gene appears to be transcribed at only about 5% the rate of the endogenous mouse beta-major globin gene, in good agreement with the relative steady state levels of the two mRNAs previously

measured for this transgenic line (20). This indicates that the hybrid beta-globin mRNA is not significantly less stable than endogenous mouse beta-globin mRNA. Therefore, the steady-state level of the hybrid globin mRNA in the erythroid tissues of any given transgenic line should directly reflect the rate of transcription of the foreign gene in that transgenic line.

To ask whether the low rate of transcription of the foreign gene might result from its failure to establish a normal "active" chromatin configuration, we performed the following analyses of DNase I hypersensitivity.

DNase I hypersensitive sites in a foreign beta-globin gene in mouse erythroid cells. This series of transgenic mouse lines was particularly suitable for the present studies of chromatin structure because the animals carried only one or a few copies of the injected gene, while the high gene copy numbers characteristic of most transgenic mice would complicate the interpretation of such experiments. The structure of the hybrid globin gene is shown in Figure 2. Also indicated, by arrows, are the positions of the major DNase I hypersensitive sites previously observed in the corresponding regions of the mouse beta-major-globin gene (7) and human beta-globin gene (3), in erythroid cells. We examined the pattern of DNase I hypersensitivity of the hybrid gene in two transgenic lines in which the gene was expressed specifically in erythroid tissues, lines 46 and 77 (refs. 17 and 20).

In erythroid tissues of adult transgenic mice in line 46, the steady-state level of hybrid beta-globin mRNA was approximately 0.6% of the endogenous beta-globin mRNA level (17). Total nucleated cells were isolated from the spleens of mice in line 46, after inducing anemia by injection of phenylhydrazine; up to 80% of the cells in such preparations are nucleated erythroid precursor cells at various stages of differentiation (31). Nuclei were prepared, and treated with varying concentrations of DNase I. The DNA was then purified, digested with restriction enzymes and analyzed by agarose gel electrophoresis and Southern blot hybridization, using a probe specific for the second intron of the human beta-globin gene (Figure 2).

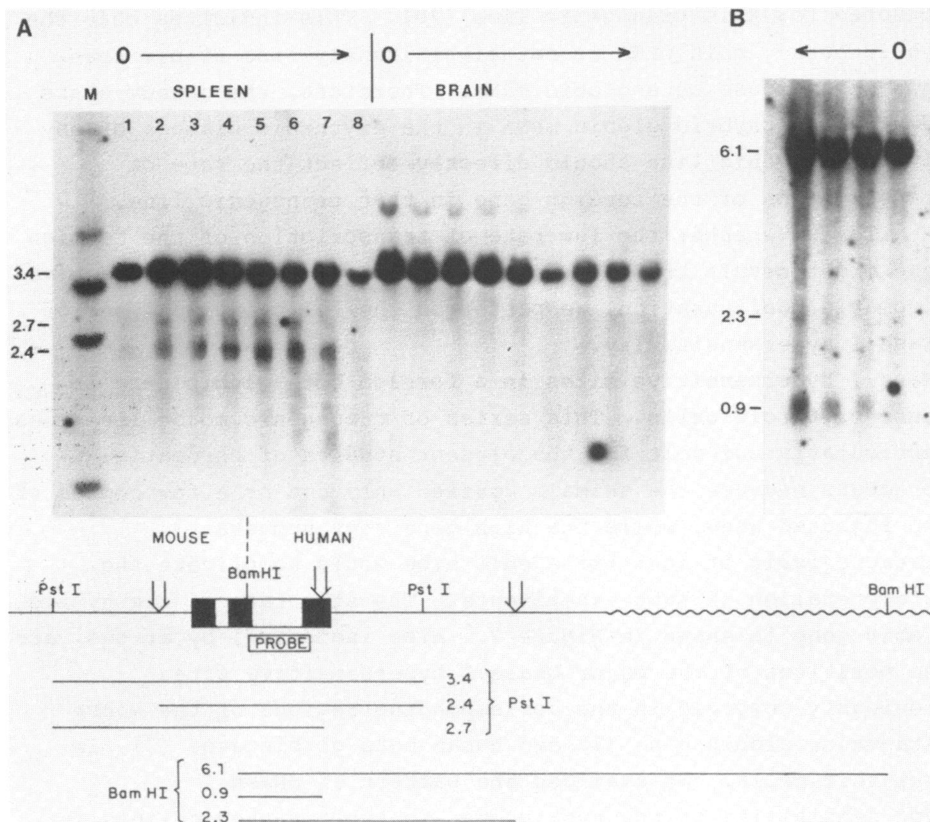

Figure 2. A foreign beta-globin gene contains appropriate DNase I hypersensitive sites in erythroid cells but not brain cells. (A) Nuclei from spleen cells after phenylhydrazine treatment (primarily erythroid cells) and brain cells from line 46 transgenic mice were digested with increasing concentrations of DNase I (direction of increasing DNase I indicated by arrows). DNA was purified, digested with PstI and analyzed by Southern blot hybridization using the probe indicated in the diagram below. M, pBR322 molecular weight markers. (B) DNA samples from DNase I treated spleen cell nuclei were digested with BamHI and analyzed as in (A). The diagram shows the location of restriction enzyme sites and DNAse I hypersensitive sites (thick arrows) in the hybrid mouse/human beta-globin gene, and a BamHI site in the flanking host DNA (wavy line). The origin of the bands produced by PstI and BamHI digestion are also indicated. Panel A is reproduced from ref. 18 by permission of Cold Spring Harbor Laboratory.

After digestion with PstI, two predominant subbands of 2.7 and 2.4 kb are seen in addition to the full length 3.4 kb PstI fragment. These subbands correspond to those that would result

from DNase I cleavage at two of the predicted hypersensitive sites: one approximately 200bp 5' to the cap site of the mouse beta-major globin gene, and another approximately 200bp 5' to the poly(A) site of the human beta-globin gene. In addition, digestion with BamHI and hybridization with the same probe yielded a major subband of 0.9 kb (Figure 2B), confirming the location of the hypersensitive site in the third exon, and eliminating any ambiguity in the origin of the 2.4 and 2.7kb bands in Figure 2A. Digestion of the DNA with BamHI also yielded a minor subband of 2.3 kb, indicating that the predicted hypersensitive site in the 3' flanking region of the human beta-globin gene (3) was also present (Figure 2B).

In transgenic line 77, the foreign gene was expressed at 2% of the endogenous level in the fetal liver but only at 0.1% of the endogenous level in adult erythroid tissues (17,20). Therefore, to examine the DNase I hypersensitivity of the hybrid beta-globin gene in this transgenic line, we used fetal livers as a source of nucleated erythroid cells. The same DNase I hypersensitive sites were observed in line 77 fetal erythroid cells as in the adult erythroid cells of line 46 mice, described above (data not shown).

Relative DNase I hypersensitivity of the foreign and endogenous beta-globin genes. As the chromatin structure of the foreign globin gene appeared to be "normal", based on location of hypersensitive sites, we sought other criteria that might distinguish it from the endogenous gene. To compare the quantitative degree of DNAse I hypersensitivity of the foreign and endogenous genes, we analyzed DNA from the same DNase-treated spleen nuclei analyzed in figure 2A, but in this case used a probe for the 5' flanking region of the endogenous beta-major globin gene (Figure 3). When the DNA was digested with BamHI, two major bands hybridized to the probe: a 5.0 kb parent band representing the full length BamHI fragment (arrow), and a 4.2 kb subband resulting from DNase I cleavage at the major 5' hypersensitive site. When the relative intensities of parent and subbands in corresponding lanes of Figures 2A and 3 were compared by densitometry, it appeared that the 5' hypersensitive site in the endogenous gene was cleaved 3-4 times more frequently than

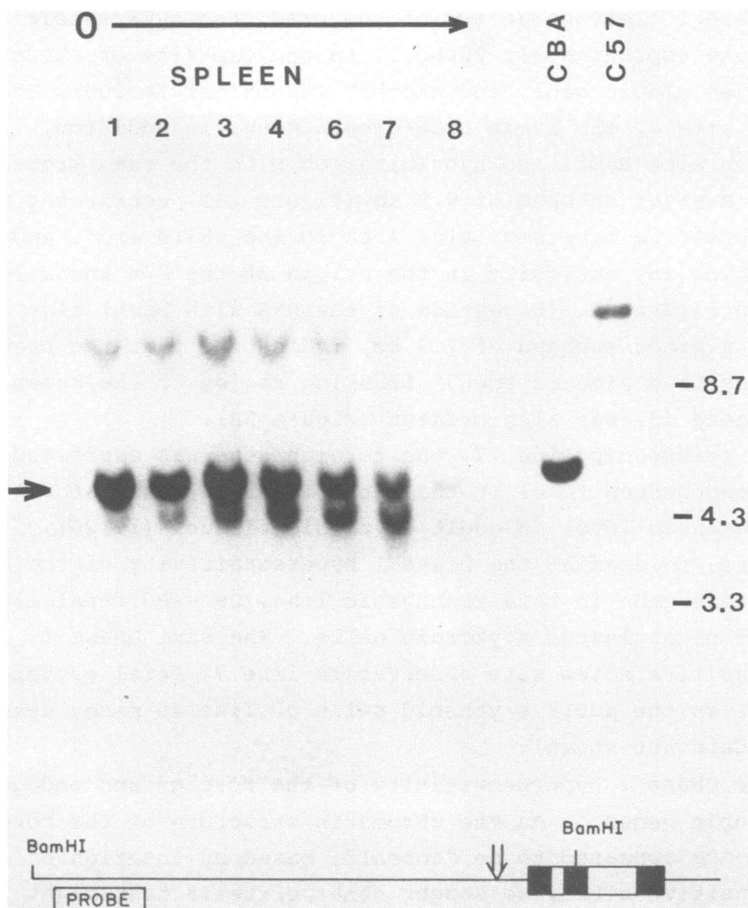

Figure 3. Analysis of DNase I cleavage at the major 5' hypersensitive site in the endogenous beta-major globin gene. The same DNase-treated spleen DNA samples analyzed in Figure 2A were digested with BamHI and analyzed by Southern blot hybridization with probe for the 5' flanking region of the endogenous beta-major globin gene, as indicated in the diagram. BamHI digestion produces a 5.0 kb parent band (solid arrow), while DNase I cleavage at the major 5' hypersensitive site (open arrow) yields a 4.2 kb subband. The relative intensity of these two bands was compared by densitometry (see text). The spleen cells used in this experiment were obtained from several transgenic mice of line 46, which were of mixed genetic background. The probe used in this experiment hybridizes primarily with the beta-major globin gene, derived from the CBA/J strain (CBA), but also cross-hybridizes weakly with a different globin gene, derived from the C57BL/6J strain (C57). The high molecular weight band seen in the transgenic spleen DNAs results from cross-hybridization with the gene of C57 origin.

the equivalent site in the foreign gene, after the same DNase treatment. The "general" DNase I sensitivity (8-10) of the foreign and endogenous genes (i.e., the rate of disappearance of the full length bands with increased DNase treatment) could not be accurately compared; such measurements require essentially pure populations of erythroid cells, and the nucleated spleen cell preparations were contaminated with non-erythroid cells.

Hypersensitive Sites in Adjacent Copies of the Foreign Gene

While line 46 mice contain a single copy of the hybrid globin gene, line 77 animals contain approximately three copies of the gene in a head-to-tail tandem array (17). While the integration of a microinjected gene in a tandem array is a common occurrence in transgenic mice (32), in most cases it is not clear whether several genes in such an array are expressed simultaneously, or whether expression is limited to a single gene. We were therefore interested to ask whether more than one copy of the hybrid globin gene might be found in an active conformation. When DNA from DNase I treated nuclei of line 77 erythroid cells was analyzed by Southern blot hybridization, a 5.2 kb subband, corresponding to the unit length of the injected DNA fragment, was released (Figure 4). This indicates that the same DNase I hypersensitive site is present in at least two adjacent copies of the foreign beta-globin gene, and suggests that at least two genes in this array may be expressed.

DNase I hypersensitive sites are tissue-specific. From the analysis of steady state mRNA levels, the hybrid beta-globin genes in lines 46 and 77 appeared not to be expressed in non-erythroid tissues (17). To determine whether this inactivity was reflected in the chromatin structure of the genes, we isolated nuclei from brain cells of adult mice in line 46, and analyzed the DNase I hypersensitivity of the foreign gene. No sub-bands were detected in either case, even after extensive DNase I digestion (Figure 2A). Similar results were obtained with brain cells from line 77 fetuses (data not shown). Thus in brain cells, the foreign beta-globin gene, like the endogenous beta-globin gene (data not shown), contains no DNase I hypersensitive sites. The general DNase I sensitivity of the foreign globin gene in erythroid vs. non-erythroid cells could

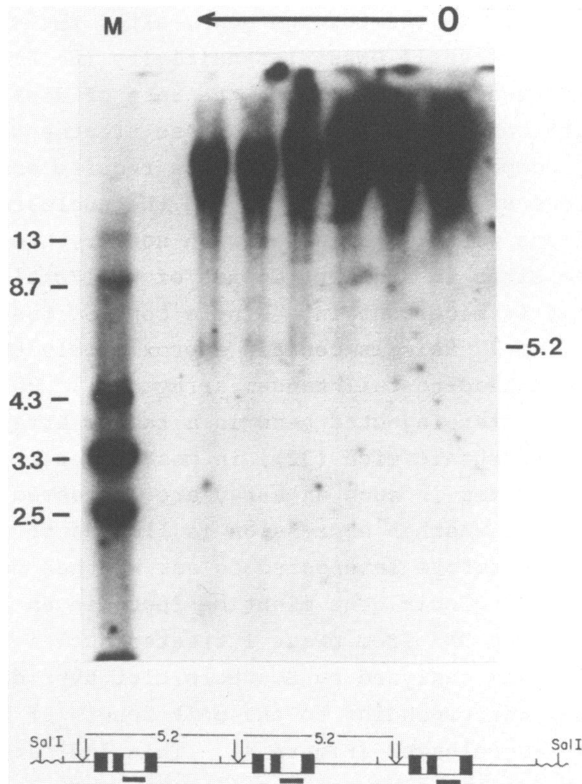

Figure 4. At least two adjacent copies of the foreign beta-globin gene in line 77 erythroid cells contain a DNase I hypersensitive site. Nuclei from day 15 fetal livers were digested with DNase I, and the DNA was purified and analyzed by Southern blot hybridization with the probe indicated. The diagram illustrates one possible origin of the observed 5.2kb sub-band: DNase I cleavage at the major 5' hypersensitive site in two (or more) adjacent copies of the gene. In the experiment shown, the DNA was also digested with Sal I, which cuts only in the host flanking DNA, to reduce its viscosity. However, the same 5.2 kb band was observed in the absence of Sal I digestion (data not shown), indicating that the band did not result from cleavage at a Sal I site. M, pBR322 molecular weight markers.

not be compared, for the reasons described above.

Absence of DNase I hypersensitive sites in a non-expressing transgenic line. In some transgenic mouse lines that contain intact copies of a foreign beta-globin gene, the gene fails to be expressed in erythroid as well as non-erythroid cells (17-19). It is assumed that this results from integration of the

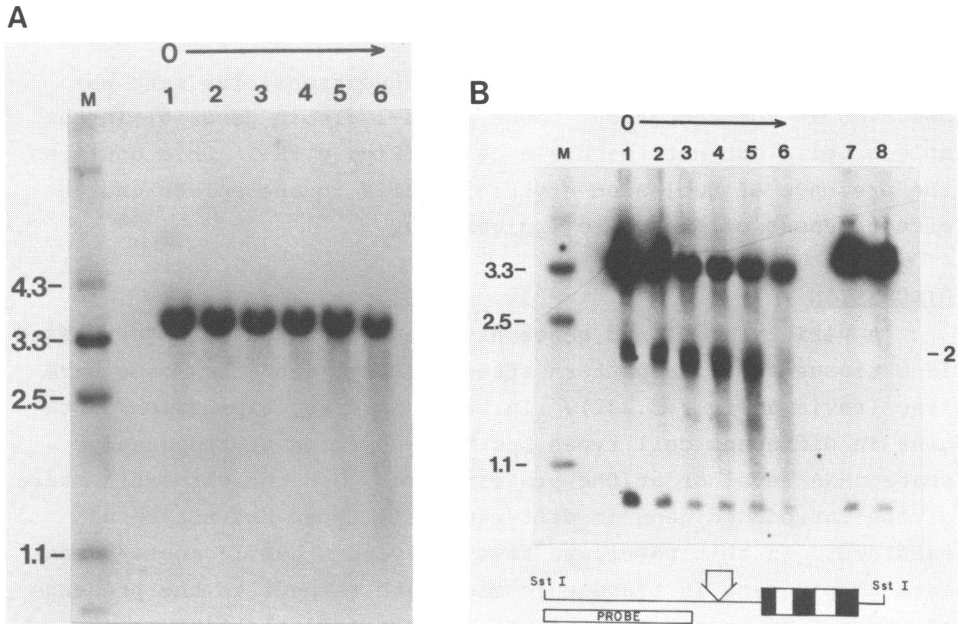

Figure 5. Absence of DNase I hypersensitive sites in a non-expressing transgenic mouse (line 47). (A) After phenylhydrazine treatment of the animal, spleen cell nuclei were digested with DNase I and the DNA was digested with PstI and analyzed as in Figure 1A. (B) The same DNase-digested spleen DNA samples as in (A) were digested with Sst I and analyzed (lanes 1-6) using a probe for the endogenous mouse alpha-1 globin gene, as shown in the diagram. The full length Sst I fragment is 3.1kb and DNase I cleavage at the 5' hypersensitive site (thick arrow) results in a 2kb subband. Brain nuclei from a line 47 mouse were similarly analyzed (lanes 7 and 8), showing the absence of DNase I hypersensitive sites in the endogenous alpha-globin gene. M, pBR322 molecular weight markers.

microinjected gene into a chromosomal position where transcription is inhibited, although the mechanism of this inhibition is unknown. Since DNase I hypersensitive sites can be detected in globin genes in the absence of transcription, under certain circumstances (15,16), we asked whether an inactive beta-globin gene in transgenic erythroid cells might, nevertheless, contain DNase I hypersensitive sites. Nucleated spleen cells as well as brain cells from a non-expressing transgenic mouse (line 47), also carrying a single copy of the hybrid beta-globin gene, were examined by the methods described above. No hypersensitive sites

were detected in the foreign gene in either cell population (Figure 5A and data not shown). In a control experiment using the same DNA samples, the expected 5' hypersensitive site was observed in the endogenous mouse alpha-1 globin gene (6) in the spleen cells but not the brain cells (Figure 5B). This confirms the presence of nucleated erythroid cells in the spleen and the effectiveness of the DNase I digestion.

DISCUSSION

A variety of cloned genes have been reported to be expressed in a tissue-specific pattern after transfer into the mouse germ line (reviewed in ref. 32). In these studies, expression of the gene in different cell types has been measured at the steady state mRNA level or at the protein level, but the chromatin state of the introduced gene in different cell types has not been examined. In this paper, we have analyzed a hybrid mouse/human beta-globin gene in transgenic mice with respect to the presence of DNase I hypersensitive sites, a characteristic feature of active globin genes.

We find that despite its insertion into foreign chromosomal positions, the cloned gene is capable of developing hypersensitive sites at the same three positions at which the hypersensitive sites are found in the corresponding portions of the endogenous mouse and human beta-globin genes. Two of the three prominent DNase I hypersensitive sites in the human beta globin gene (those in the third exon and 3' flanking region; ref. 3) have not been observed in the endogenous mouse beta-major globin gene (7), yet they are found in the human portion of the hybrid globin gene in transgenic mouse erythroid cells. This suggests that differences in the mouse and human DNA sequences, rather than factors present in human but not mouse erythroid cells, are responsible for the additional hypersensitive sites in the human gene. The dominant role of DNA secondary structure, and hence DNA sequence, in determining the positions of DNase I and S1 nuclease hypersensitive sites has been previously described (26), and our results support this idea.

Just as the potential of an endogenous globin gene to form DNase I hypersensitive structures is suppressed in non-erythroid

cells, the foreign globin gene remains insensitive to DNase I in brain cells. Thus, the developmental processes that render the endogenous globin gene inaccessible to DNase I cleavage in non-erythroid cells appear to operate on a foreign globin gene. In this respect, a foreign gene carried in the mouse germ line differs significantly from one introduced into cultured cells by co-transformation with a selectable marker. Cloned genes introduced into cells by transfection are usually found in an open or "derepressed" state, with respect to both chromatin structure and accessibility to positive trans-activators, even in inappropriate cell types (23-26). This may be a consequence of the "co-transformation" procedure (33), in which cells are selected for expression of a marker gene, perhaps favoring integration of the foreign DNA into open regions of chromatin. Alternatively, the formation of inactive chromatin may occur in specific cell lineages at an early stage in cell differentiation, and genes transfected into cultured cell lines may not be exposed to these early events. In contrast, the use of transgenic mice may now provide a means to investigate the control of tissue-specific alterations in chromatin structure during development.

Although the foreign globin gene in mouse erythroid cells develops hypersensitive sites in the correct locations, its level of expression in these cells is at most a few percent of the endogenous level. In fetal liver cells of line 77 mice, we directly confirmed, by nuclear run-off analysis, that this reflects a low rate of transcription of the foreign gene rather than a low stability of the hybrid mouse/human beta-globin mRNA. Because the stability of the hybrid mRNA is unlikely to differ between different transgenic lines, we infer that the low steady state mRNA level in the erythroid tissues of line 46 mice (17) also reflects a low rate of transcription. Thus, the alterations in chromatin structure that result in DNase I hypersensitivity at appropriate sites in an exogenous beta-globin gene are not sufficient to ensure a maximal rate of transcription. A similar lack of correlation between the establishment of hypersensitive sites and the level of expression has been observed for the human beta-globin gene _in vivo_ (3). While the level of expression of this gene is 10-20 times lower in fetal liver than in adult bone

marrow erythroid cells, the gene contains DNase I hypersensitive sites at the same positions in both erythroid cell populations.

Although the pattern of DNase I hypersensitive sites in the mouse/human hybrid gene was qualitatively normal, we were able to detect a quantitative difference between the hybrid gene and the endogenous beta-major globin gene. This comparison was made using line 46 transgenic mice, which carry a single copy of the hybrid beta-globin gene. The 5' (-200) region of the hybrid gene appeared to be cleaved approximately 3-4 times less frequently than the same site in the endogenous beta-major globin gene, during the same exposure to DNase I. One possible interpretation is that the 5' site in the hybrid gene is less sensitive to DNase I than the same site in the endogenous gene, due to a subtle difference in chromatin structure. A "weak" 5' hypersensitive site might reflect the binding of some, but not all, of the protein factors that are capable of binding at that region, which could coincidently be responsible for the observed low rate of transcription. An alternative explanation, which cannot be excluded, is that the 5' site in the hybrid gene is maximally hypersensitive in some cells but insensitive to DNase in a majority of cells within the erythroid cell population analyzed, while the endogenous gene is hypersensitive in a larger proportion of the cells.

The total lack of expression of the hybrid globin gene at the mRNA level in line 47 mice is reflected in a corresponding absence of DNase I hypersensitive sites in erythroid cells from these animals. Apparently, the presence of an intact globin gene in developing erythroid cells is not a sufficient condition for the gene to assume an active chromatin configuration. These results do not shed light on the mechanism of inhibition, but are consistent with the possibility that in this transgenic line, and in other non-expressing lines, the gene has integrated in a region of chromatin that is "closed" in erythroid cells.

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