MOLECULAR AND CELLULAR BIOLOGY, Oct. 1989, p. 4581–4584
0270-7306/89/104581-04$02.00/0

Vol. 9, No. 10

β-Globin Enhancers Target Expression of a Heterologous Gene to Erythroid Tissues of Transgenic Mice

JEANNE MAGRAM,† KAREN NIEDERREITHER,‡ AND FRANK COSTANTINI*

Department of Genetics and Development, College of Physicians and Surgeons, Columbia University, 701 West 168 Street, New York, New York 10032

Received 25 April 1989/Accepted 14 July 1989

To examine the role of human β-globin enhancers in tissue-specific and developmental regulation, a hybrid β-globin–simian virus 40 gene was analyzed in transgenic mice. A β-globin DNA fragment containing two previously defined enhancers stimulated transcription from the simian virus 40 promoter in a tissue- and stage-specific pattern similar to that of the normal β-globin gene. These results help to define the functions of β-globin regulatory elements and suggest an approach for targeted expression of heterologous genes in erythroid cells in vivo.

The human adult β-globin gene is subject to both tissue-specific and developmental regulation. When transferred with limited amounts of flanking DNA into the genomes of transgenic mice (4, 9, 15, 19) or cultured erythroid cells (5, 6, 23, 24), the β-globin gene displays an appropriate tissue-specific and developmental-stage-specific pattern of expression. Thus, *cis*-acting sequences sufficient to dictate a qualitatively normal pattern of expression have been shown to reside close to or within the gene. Recent studies have shown that these elements include two enhancers, one in the 3′-flanking region and one within the gene, as well as regulatory elements in the promoter region (1, 2, 12, 20, 21). In addition, more distant DNA sequences upstream from the β-globin gene cluster have been shown to play an important role in the quantitative transcriptional regulation of the β-globin gene and presumably of other members of the gene family (10, 17, 18, 22).

The sequences most clearly shown to play a role in developmental regulation are the intragenic and 3′ enhancers, which were initially defined through studies employing transgenic mice (2, 12, 20, 21). Sequences with enhancer activity included a region approximately 600 to 900 base pairs 3′ to the poly(A) site and a region which includes parts of the second intron and third exon. Both of these enhancers have also been shown to be involved in the induction of β-globin gene transcription during the in vitro differentiation of murine erythroleukemia cells (1).

In this study, we examined the ability of the β-globin enhancers to direct the tissue-specific and stage-specific transcription from a heterologous, i.e., nonglobin, promoter in transgenic mice. These experiments addressed several questions left unresolved by previous studies. First, we wanted to definitively test whether the β-globin enhancers are erythroid cell-specific in their activity. Second, we wanted to determine whether the β-globin enhancers determine the stage-specificity of the β-globin gene, i.e., whether they are active solely in fetal and adult and not embryonic erythroid cells. Finally, we were interested in the possibility that the β-globin enhancers might be useful in future experiments to target the expression of heterologous genes of interest to erythroid cells.

Tissue-specific expression of the SV40 early region under the control of β-globin enhancers. The β-globin DNA fragment we chose to test was a 2.8-kilobase *Bam*HI-*Xba*I fragment including the second intron, the third exon, and 1.5 kilobases of 3′-flanking DNA. This DNA fragment contains both of the previously characterized enhancers and can activate the otherwise silent γ-globin gene (3, 13) in fetal and adult erythroid cells (2; unpublished data). The *Bam*HI-*Xba*I fragment was joined to a DNA fragment from the simian virus 40 (SV40) early region, which contained the early promoter but lacked a complete copy of the SV40 enhancer and was therefore expected to be transcriptionally inactive in the absence of an added enhancer. In addition, the SV40 DNA contained a deletion in the sequence encoding large T antigen which eliminated the transforming effect of large T antigen (7). The β-globin–SV40 construct (β3′tSV40) and a control SV40 construct lacking β-globin sequences (tSV40) are shown in Fig. 1.

In the first experiment, DNAs were microinjected into (CBA/J × C57BL/6J)F_1 mouse zygotes (11), and the developing fetuses were analyzed on day 16 of gestation. Transgenic fetuses were identified by Southern analysis (not shown), total RNA was isolated from several tissues, and SV40 transcripts were detected by RNase protection analysis (Fig. 1). In six transgenic fetuses carrying the control construct tSV40, no expression was observed in any tissue examined as expected (Fig. 2 and data not shown). In contrast, two fetuses (B8 and B26) carrying the β3′tSV40 transgene contained SV40 RNA in the liver, the major erythropoietic tissue at this stage of development, but not in the four nonerythroid tissues examined. The levels of expression of this hybrid gene could not be compared with levels of expression of the normal β-globin gene because the relative stabilities of β-globin and SV40 mRNAs in mouse tissues are not known. The other five transgenic fetuses contained no detectable SV40 transcripts (Fig. 2 and data not shown).

To test whether the β-globin DNA fragment also acts as an enhancer in adult erythroid cells, we generated a second series of transgenic mice carrying β3′tSV40. Seven founder transgenic mice were obtained, and four of these produced transgenic offspring. Animals in two of the four lines (lines

* Corresponding author.
† Present address: Department of Microbiology and Immunology and the G. W. Hooper Foundation, University of California Medical Center, San Francisco, CA 94143.
‡ Present address: Department of Molecular Genetics, M. D. Anderson Hospital and Tumor Institute, Houston, TX 77030.

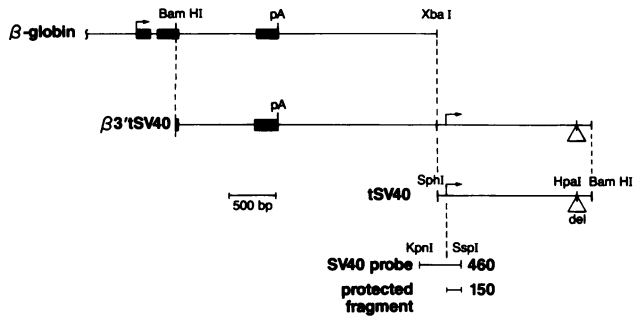

FIG. 1. β-Globin–SV40 fusion gene and probe for RNase protection analysis. A *Bam*HI-*Xba*I fragment of the human β-globin gene and a *Sph*I-*Bam*HI fragment of SV40 DNA (tSV40) were joined as indicated to generate the fusion gene (β3'tSV40). Solid boxes indicate β-globin exons; arrows indicate the transcription initiation sites of the β-globin gene and the SV40 early region. The tSV40 fragment contains a deletion of sequences between two *Hpa*I sites (positions 2666 and 3733) and lacks a complete copy of the 72-base-pair (bp) repeat. The β3'tSV40 fragment for microinjection was excised from plasmid sequences by digestion with *Bam*HI, whereas the tSV40 control fragment was excised with *Sph*I and *Bam*HI. The *Kpn*I-*Ssp*I fragment was subcloned into the plasmid pSP64 (16) and used to synthesize an RNA probe (SV40 probe) for RNase protection analysis.

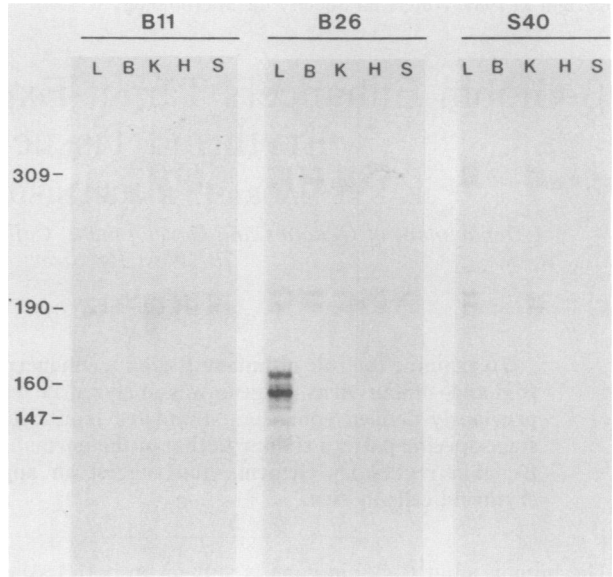

FIG. 2. Tissue-specific expression of construct β3'tSV40 in a transgenic fetus. RNA was isolated (21) from liver (L), brain (B), kidney (K), heart (H), and skin (S) of 16-day transgenic fetuses and hybridized with a ^{32}P-labeled RNA probe (SV40 probe, Fig. 1), and the products were digested with RNase and analyzed by polyacrylamide gel electrophoresis and autoradiography (16). B11 and B26 are transgenic fetuses carrying the β3'tSV40 construct, whereas S40 is a transgenic fetus carrying the tSV40 control construct. The bands in lane L of fetus B26 approximately 150 nucleotides long, represent transcripts initiated at the SV40 early cap site, while the faint bands at approximately 200 and 300 nucleotides in all the lanes are artifacts of the RNase protection assay. Either 10 μg (liver, brain, and skin) or 2 μg (kidney and heart) of total RNA was used for each reaction. Although the sensitivity of the assay was therefore lower for kidney and heart RNAs, no specific protected fragments were seen even after very long exposure of the autoradiogram. All RNA samples were undegraded as judged by their electrophoretic profiles on formaldehyde agarose gels. Nucleotide numbers are shown on the left.

20 and 30) produced SV40 transcripts in peripheral blood and bone marrow (data not shown). To examine the tissue specificity of expression, an additional mouse in each line was treated with phenylhydrazine to induce anemia and consequent erythropoiesis in the spleen (8), and total RNA was isolated from the spleen and four nonerythroid tissues. The transgene was found to be expressed predominantly in the spleen in transgenic lines 20 and 30 (Fig. 3).

The β-globin 3' fragment acts as a developmental stage-specific enhancer. To examine the role of β-globin internal and 3' elements in stage-specific expression, we compared the expression of the β3'tSV40 transgene in erythroid tissues at different stages of development. We examined RNA from day 11.5 embryonic blood cells, day 16 fetal livers, and adult anemic spleens. SV40 early RNA was detected in the fetal livers and the adult spleens but not in embryonic blood from transgenic line 20 or 30 (Fig. 4). The integrity of the embryonic blood cell RNA was demonstrated by hybridization with a probe specific for the endogenous embryonic βh1 globin gene (Fig. 4, bottom). Thus, the β3'tSV40 construct, like the endogenous murine β-globin genes, is specifically active in fetal and adult but not embryonic erythroid cells.

Discussion. These experiments demonstrate that the human adult β-globin gene contains a tissue-specific and developmental stage-specific enhancer that can function in association with the heterologous SV40 early promoter. While previous studies have identified two distinct enhancers within the *Bam*HI-*Xba*I fragment (1, 2, 12, 20, 21), it has not been established to what extent either of these enhancers is responsible for the erythroid-tissue-specific expression of the β-globin gene. Although it was shown that constructs containing one or both of the β-globin enhancers joined to the γ-globin gene were expressed specifically in erythroid tissues of transgenic mice, this could have been due in part to regulatory elements contributed by the γ-globin gene. A limited degree of tissue specificity has been demonstrated for a construct containing the β-globin 3' enhancer fused to the H2-K^{bm1} gene (1, 12). In the present study, we examined a wider variety of tissues in both fetal and adult mice carrying a β-globin–SV40 fusion gene and have observed a considerable degree of erythroid-tissue specificity. Our data indicate that sequences within the *Bam*HI-*Xba*I fragment presumably (but not necessarily) corresponding to one or both of the previously identified enhancers are important elements for the tissue-specific expression of the β-globin gene. Our findings also argue against the possibility that promoter or upstream regulatory elements of the β-globin gene are necessary for erythroid-cell-specific expression, consistent with previous studies (19, 21).

One potential application of these results may be the ability to target the expression of various heterologous genes to the erythroid cells of transgenic mice. While our results indicate that β-globin enhancers can confer tissue specificity to heterologous genes, they do not establish the levels of expression that may be achieved. This is likely to depend on the promoter as well as other factors such as the stability or translational efficiency of different mRNAs in erythroid cells. It is likely that the addition of dominant control region sequences (18, 22) located upstream from the β-globin gene cluster could augment the level and the uniformity of expression of such constructs, as dominant control region sequences do when added to the β-globin gene itself (10, 18, 22). However, it has not yet been determined whether the dominant control region alone can target the expression of

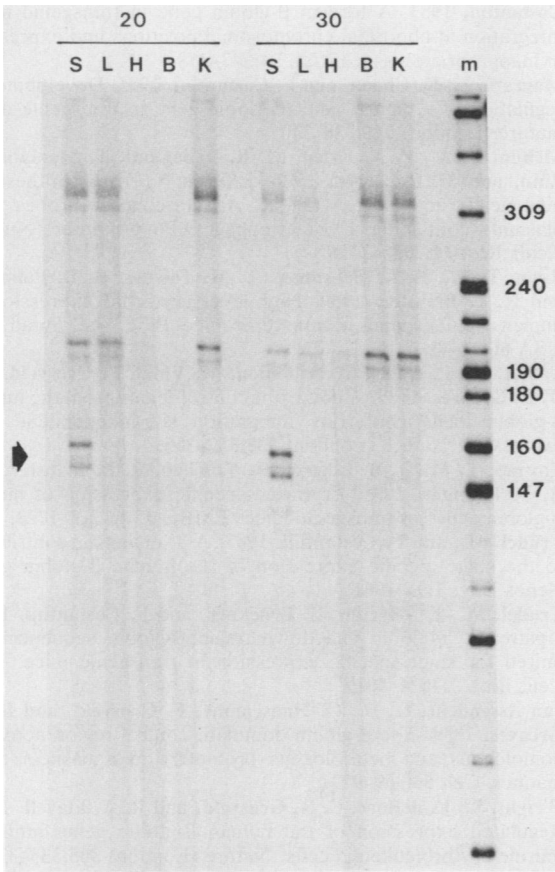

FIG. 3. Tissue-specific expression of construct β3′tSV40 in adult mice of transgenic lines 20 and 30. Transgenic offspring in lines 20 and 30 were treated with phenylhydrazine to induce splenic erythropoiesis (8, 14), and RNA was isolated from the spleen (S), liver (L), heart (H), brain (B), and kidney (K) and analyzed as described in the legends to Fig. 1 and 2. m, Molecular weight markers (pBR322 DNA digested with *Hpa*II). Twenty micrograms of total RNA from each tissue was used for analysis. The bands approximately 150 nucleotides long represent transcripts initiated at the SV40 promoter. All RNA samples were undegraded as judged by their electrophoretic profiles on formaldehyde agarose gels. Nucleotide numbers are shown on the right.

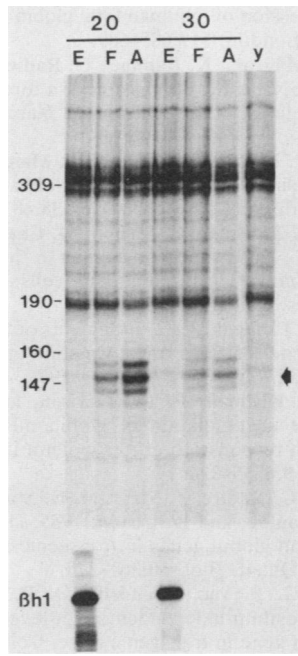

FIG. 4. Developmental stage-specific expression of construct β3′tSV40 in transgenic lines 20 and 30. Total RNA was isolated from blood cells of transgenic embryos on day 11.5 of gestation (E), livers of transgenic fetuses on day 16 of gestation (F), and adult transgenic mouse spleens after treatment with phenylhydrazine (A) (8, 14). y, Yeast RNA. (Top) Ten micrograms of each kind of RNA was analyzed by an RNase protection assay with the SV40 probe. Approximately one-third of sample 30A was lost while loading the gel. The faint band at 150 nucleotides seen in the embryonic RNA samples is also seen in the yeast RNA sample and is therefore a background band. (Bottom) Analysis of the same RNAs (10 μg of each) with an RNA probe specific for the mouse endogenous βh1 embryonic globin mRNA (3). The 195-nucleotide fragment protected by βh1 mRNA is shown. Nucleotide numbers are shown on the left.

nonglobin genes in an erythroid-tissue-specific pattern in transgenic mice.

Previous experiments have not established whether the β-globin enhancers are active in erythroid cells at a specific stage or at all stages of development. Although intact β-globin transgenes are not expressed at the embryonic stage (9, 15, 19), this could be due either to failure of the enhancers to stimulate transcription in the embryonic blood cells or to negative regulation mediated by some other element, e.g., a promoter or upstream element. In the present study, we have shown that transcription of the SV40 early promoter is activated by the β-globin enhancers only in fetal and adult and not embryonic erythroid cells. This supports the idea that one or both of the β-globin enhancers are responsible for the developmental-stage specificity as well as the tissue specificity of the β-globin gene. Surprisingly, we have recently found that under some circumstances the β-globin 3′-flanking enhancer appears to function in embryonic erythroid cells. The human β-globin promoter can be activated in mouse embryonic erythroid cells when placed adjacent to upstream elements from the $^{G}\gamma$-globin gene; when the β-globin 3′-flanking enhancer was deleted from such a construct but the internal enhancer was retained, a substantial reduction in the average level of expression was observed (C. Perez-Stable and F. Costantini, manuscript in preparation). This suggests that the β-globin 3′-flanking enhancer is indeed capable of stimulating expression in embryonic blood cells when combined with γ-globin upstream sequences. Together with our present results, this raises the possibility that the intragenic enhancer (or some element other than the 3′ enhancer) may determine specificity for fetal- and adult-stage erythroid cells.

This work was supported by Public Health Service grants HD-17704 and HL-28381 to F.C. from the National Institutes of Health, grant 1-1016 from the March of Dimes Birth Defects Foundation, and a grant from the Pew Scholars Program in the Biomedical Sciences.

LITERATURE CITED

1. **Antoniou, M., E. DeBoer, G. Habets, and F. Grosveld.** 1988. The human β-globin gene contains multiple regulatory regions: identification of one promoter and two downstream enhancers. EMBO J. 7:377–384.
2. **Behringer, R. R., R. E. Hammer, R. L. Brinster, R. D. Palmiter, and T. M. Townes.** 1987. Two 3′ sequences direct adult erythroid specific expression of human β-globin genes in transgenic mice. Proc. Natl. Acad. Sci. USA **84**:7056–7060.
3. **Chada, K., J. Magram, and F. Costantini.** 1986. An embryonic

pattern of expression of a human fetal globin gene in transgenic mice. Nature (London) **319**:685–689.

4. **Chada, K., J. Magram, K. Raphael, G. Radice, E. Lacy, and F. Costantini.** 1985. Specific expression of a foreign β-globin gene in erythroid cells of transgenic mice. Nature (London) **314**: 377–380.
5. **Chao, M. V., P. Mellon, P. Charnay, T. Maniatis, and R. Axel.** 1983. The regulated expression of β-globin genes introduced into mouse erythroleukemia cells. Cell **38**:483–493.
6. **Charnay, P., R. Treisman, P. Mellon, M. Chao, R. Axel, and T. Maniatis.** 1984. Differences in human α and β-globin gene expression in mouse erythroleukemia cells: the role of intragenic sequences. Cell **38**:251–263.
7. **Colby, W., and T. Shenk.** 1982. Fragments of the simian virus 40 transforming gene facilitate transformation of rat embryo cells. Proc. Natl. Acad. Sci. USA **79**:5189–5193.
8. **Conkie, D., L. Kleiman, P. R. Harrison, and J. Paul.** 1975. Increase in the accumulation of globin mRNA in immature erythroblasts in response to erythropoietin in vivo or in vitro. Exp. Cell Res. **93**:315–324.
9. **Costantini, F., G. Radice, J. Magram, G. Stamatoyannopoulos, T. Papayannopoulou, and K. Chada.** 1985. Developmental regulation of human globin genes in transgenic mice. Cold Spring Harbor Symp. Quant. Biol. **50**:361–370.
10. **Grosveld, F., G. B. van Assendelft, D. R. Greaves, and G. Kollias.** 1987. Position-independent, high-level expression of the human β-globin gene in transgenic mice. Cell **51**:975–985.
11. **Hogan, B. L. M., F. Costantini, and E. Lacy.** 1986. Manipulating the mouse embryo: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
12. **Kollias, G., J. Hurst, E. DeBoer, and F. Grosveld.** 1987. A tissue and developmental specific enhancer is located downstream from the human β-globin gene. Nucleic Acids Res. **15**:5739–5747.
13. **Kollias, G., N. Wrighton, J. Hurst, and F. Grosveld.** 1986. Regulated expression of human $^{A}\gamma$-, β-, and hybrid γβ-globin genes in transgenic mice: manipulation of the developmental expression patterns. Cell **46**:89–94.
14. **Lacy, E., S. Roberts, E. P. Evans, M. D. Burtenshaw, and F. D. Costantini.** 1983. A foreign β-globin gene in transgenic mice: integration at abnormal chromosomal positions and expression in inappropriate tissues. Cell **34**:343–358.
15. **Magram, J., K. Chada, and F. Costantini.** 1985. Developmental regulation of a cloned adult β-globin gene in transgenic mice. Nature (London) **315**:338–340.
16. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, D. Zinn, and M. R. Green.** 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7056.
17. **Ryan, T. M., R. R. Behringer, T. M. Townes, R. D. Palmiter, and R. L. Brinster.** 1989. High-level erythroid expression of human α-globin genes in transgenic mice. Proc. Natl. Acad. Sci. USA **86**:37–41.
18. **Talbot, D., P. Collis, M. Antoniou, M. Vidal, F. Grosveld, and D. R. Greaves.** 1989. A dominant control region from the human β-globin locus conferring integration site-independent gene expression. Nature (London) **338**:352–355.
19. **Townes, T. M., J. B. Lingrel, H. Y. Chen, R. L. Brinster, and R. D. Palmiter.** 1985. Erythroid-specific expression of human β-globin genes in transgenic mice. EMBO J. **4**:1715–1723.
20. **Trudel, M., and F. Costantini.** 1987. A 3′ enhancer contributes to the stage-specific expression of the human β-globin gene. Genes Dev. **1**:954–961.
21. **Trudel, M., J. Magram, L. Bruckner, and F. Costantini.** 1987. Upstream $^{G}\gamma$-globin and downstream β-globin sequences required for stage-specific expression in transgenic mice. Mol. Cell. Biol. **7**:4024–4029.
22. **van Assendelft, G. B., O. Hanscombe, F. Grosveld, and D. R. Greaves.** 1989. The β-globin dominant control region activates homologous and heterologous promoters in a tissue-specific manner. Cell **56**:969–977.
23. **Wright, S., E. deBoer, F. G. Grosveld, and R. A. Flavell.** 1983. Regulated expression of the human β-globin gene family in murine erythroleukemia cells. Nature (London) **305**:333–336.
24. **Wright, S., A. Rosenthal, R. Flavell, and F. Grosveld.** 1984. DNA sequences required for regulated expression of globin genes in murine erythroleukemia cells. Cell **38**:265–273.