Vol. 150, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1988 Pages 204-211

REGULATION OF MURINE α-, βMAJOR-, and βMINOR-GLOBIN GENE EXPRESSION

Nadine Weich, Paul A. Marks, and Richard A. Rifkind

Dewitt Wallace Research Laboratories
Memorial Sloan-Kettering and the Sloan-Kettering Division of the
Graduate School of Medical Sciences, Cornell University
New York, New York 10021

Received November 6, 1987

SUMMARY: Transcriptional properties of the mouse globin genes and their promoters were examined in COS cells using transient expression assays. Transfected intact mouse α_1-globin genes generate transcripts, whereas βmaj and βmin globin transcripts are detected only if the genes are associated with a strong exogenous (SV40) enhancer sequence. Under these conditions the ratio of accumulated βmaj and βmin mRNA sequences approximates the 4:1 ratio observed in reticulocytes and murine erythroleukemia cells (MELC) induced to differentiate by hexamethylene bisacetamide (HMBA). As determined using hybrid genes consisting of globin gene 5' regions fused to the chloramphenicol acetyltransferase (CAT) structural gene, this 4:1 ratio appears dependent upon the relative activities of the two 5' promoter regions.

Mechanisms involved in controlling expression of the mouse α_1- and the two linked adult β-globin genes (βmaj and βmin) have been examined in differentiation-inducible MELC (1). Although the mechanisms are not fully understood, there is evidence for differences in regulation of mouse α_1, βmaj, and βmin genes (2,3). To gain further understanding of regulated globin gene expression in the mouse we have examined their transcriptional properties.

Transient expression assays have suggested differences in transcriptional control among the human globin genes (4-6). In the present studies transient expression was used to investigate (1) the transcriptional properties of cloned intact mouse α_1-, βmaj- and βmin-globin genes, and (2) to examine their promoters, which differ in sequence and transcriptional activity (2,3,7). To accomplish the second objective, α_1, βmaj and βmin 5' regulatory sequences were fused to the CAT structural gene and the influence of each measured as CAT enzymatic activity (8). The results suggest that different mechanisms are responsible for

0006-291X/88 $1.50

transcriptional control of the mouse α_1- and β-globin genes. Following transfection, α_1-globin mRNA is detected, whereas the βmaj and βmin genes require an exogenous enhancer. The relative levels of βmaj and βmin mRNA reflect closely their relative expression in HMBA-induced MELC and in normal erythroid cells. Each level of expression appears determined, at least in part, by sequences 5' to the structural genes.

Materials and Methods

Globin and hybrid gene plasmids. Plasmids were prepared by alkaline lysis and two cesium chloride-ethidium bromide equilibrium centrifugations (9,10). Recombinant PSV_0-cat, lacking the SV40 early transcription unit, was obtained from PSV_2-cat as described (8). PSV_2-cat containing the SV40 early transcription unit was the source of SV40 DNA sequences. A 198 bp fragment containing the origin of replication and a 216 bp fragment containing the 72 bp tandem repeats were ligated into the BamHI sites of plasmids pBR327 (11) and PSV_0-cat. Recombinants pBR327SH and PSV_{SH}-cat consist of pBR327 or PSV_0-cat sequences and the SV40 origin of replication. Recombinants pBR327NP and PSV_{NP}-cat contain the SV40 transcription enhancer. A 3 kb SacI fragment containing the α_1-globin gene (12), a 7 kb EcoRI fragment containing the βmaj-globin gene (13), and a 6.2 kb EcoRI-HindIII βmin fragment (13) were inserted into each pBR327 plasmid (Fig. 1A). The globin regulatory sequences(GRS) (5' promoter and cap sites) studied were: a 700 bp NcoI α_1 fragment; a 550 bp HincII βmaj fragment; and a 600 bp BamHI-HincII βmin fragment. These were inserted into the HindIII site of each PSV-cat plasmid (Fig. 1B).

DNA transfection and CAT assay. One day before transfection, COS cells, in Dulbecco's modified Eagle's medium and 10% fetal calf serum, were plated ($4x10^4$ cells/cm^2). For transfection, 10μg of plasmid DNA added to $CaCl_2$ and Hepes buffer containing phosphate (14), was then added to the cells. After four hours exogenous DNA was removed and Hepes-buffered 15% glycerol was layered over the cells for 1 min. Cells were harvested for RNA preparation and CAT assay (8) 48 hours after transfection.

RNA Preparation and RNA Probes. DBA/2 mice were injected intraperitoneally with 0.1 ml 1% phenylhydrazine on days 0, 4 and 7, and bled on day 9. Red cells were washed in phosphate buffered saline and total RNA prepared (15,16). MELC DS-19 (subclone 9) cells were maintained in medium lacking nucleosides (Gibco Laboratories) with 10% fetal calf serum. Cells were induced with 5mM HMBA (96 hrs), and total RNA prepared. To construct templates for complementary strand RNA probes a pGEM4 vector (Promega Biotec) containing the Sp6 (17) and T_7 promoters flanking a pUC18 multiple cloning region was used. Probes α_1-pGEM4 and α_1X1-pGEM4 were linearized with HindIII prior to transcription from the Sp6 promoter. βmaj-pGEM4 (T_7 promoter) and βmin-pGEM4 (Sp6) were linearized with EcoRI and HindIII, respectively. The probes were synthesized as suggested by Promega Biotec except that α^{32}P-UTP (3000 Ci/mmole) was substituted for α^{32}P-CTP. Transcription was at 37°C and the hybridization and RNase protection assays performed as described (17).

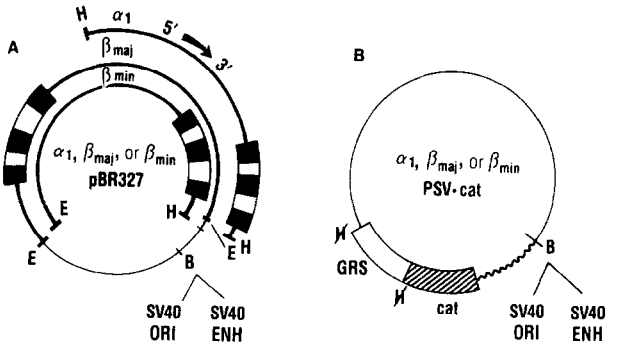

Figure 1. Globin gene and globin-CAT hybrid gene plasmids. (A) α_1, βmaj, or βmin pBR327 constructs: the arrow represents direction of transcription, the thin line, sequences from pBR322; the thick line, mouse sequences flanking the globin genes; ORI, origin of replication; ENH, transcription enhancer. (B) α_1, βmaj, or βminPSV-cat constructs: GRS represents globin 5' regulatory sequences. The slash through restriction endonuclease cleavage sites indicate loss of that site during plasmid construction (B-BamHI; E-EcoRI; H-HindIII).

Results and Discussion

Globin gene expression in mouse cells

Accumulation of globin mRNA was determined by RNase protection with uniformly labeled RNA probes (Fig. 2 and 3). Although βmaj and βmin homology is about 98%, the RNase protection assay can detect non-homology of 4 consecutive bases. For example, when the

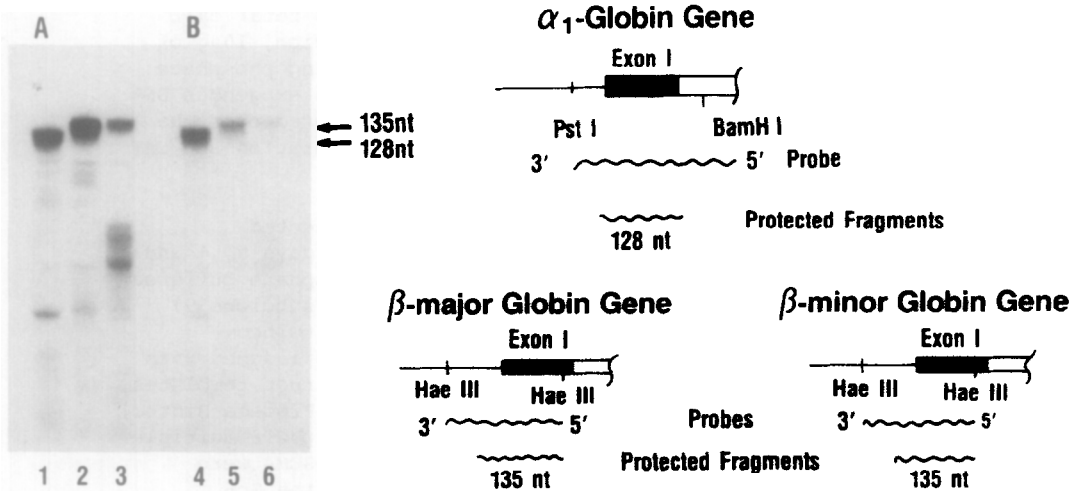

Figure 2. RNase protection analysis of total RNA from DBA/2 mouse reticulocytes and MELC. Protected RNA fragments were analyzed on an 8% polyacrylamide -8M urea gel. The RNA probes used are shown in the diagram. (A) Reticulocyte RNA (1μg): (Lane 1) α; (lane 2) βmaj; and (lane 3) βmin. (B) HMBA-induced MELC RNA (5μg): (Lane 4) α_1; (lane 5) βmaj; (lane 6) βmin.

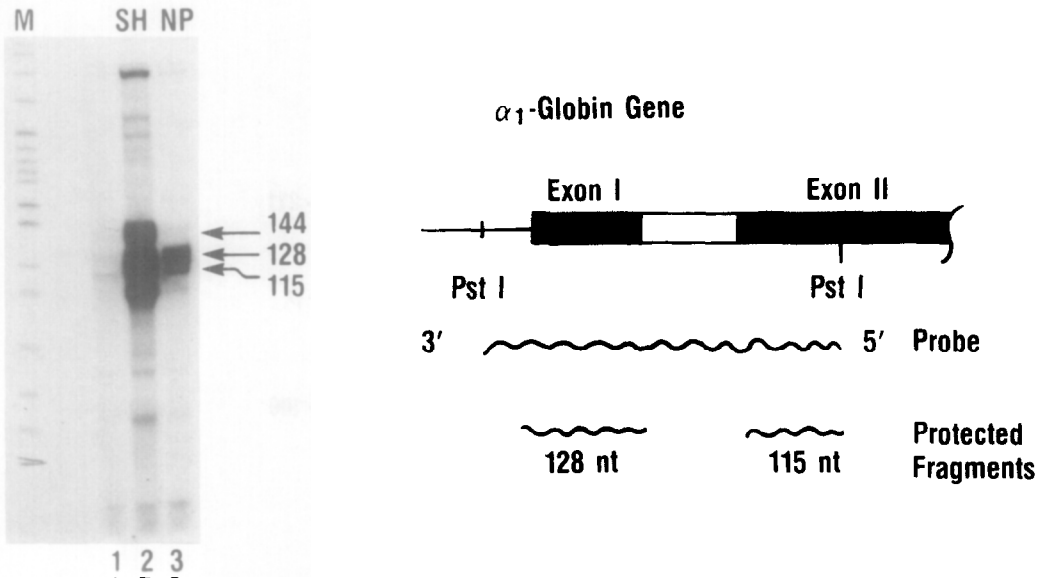

Figure 3. RNA from cells transfected with α_1-globin gene. Cells were transfected with 10μg DNA. α_1pBR327 (lane 1), α_1pBR327SH (lane 2), or α_1pBR327NP (lane 3). 20μg RNA (lanes 1 & 3) or 6μg RNA (lane 2) were analyzed by RNase protection, on an 8% polyacrylamide -8M urea gel. The α_1 probe used in this experiment is shown in the diagram. 144nt fragments are produced from incorrectly initiated and/or spliced globin mRNA. Correctly processed mRNA yields 128nt and 115nt fragments. Lane M: size markers from ^{32}P-labeled pBR322 DNA cut with MspI.

βmaj probe is hybridized with βmin mRNA and treated with RNase the full length 135nt fragment is not detected; instead, digestion occurs at several sites of non-homology and smaller fragments are detected.

RNA from DBA/2 mouse reticulocytes (Fig. 2, lanes 1, 2 and 3), or from MELC exposed to HMBA for 96 hrs (Fig. 2, lanes 4, 5 and 6), was assayed for α_1, βmaj and βmin sequences. The ratio of βmaj to βmin mRNA was 4:1 in RNA from both sources. The ratio of α to β mRNA in the not yet fully mature HMBA-induced MELC was 2:1 (18,19) and 1:1 in mature reticulocytes.

Globin gene expression in COS cells

To study the effect of regulatory regions on transcription of intact mouse α_1-, βmaj-, and βmin-globin genes, we constructed two series of plasmids that were assayed for transient expression. In each series, for each gene (Fig. 1A) or each 5' sequence (Fig. 1B), one construct contains the SV40 origin of replication (ori) for sequence amplification (20), to insure sensitivity for expression. Since the SV40 enhancer sequence markedly stimulates transcription of some heterologous genes in COS cells (5,21,22), each series also contains constructs with that enhancer.

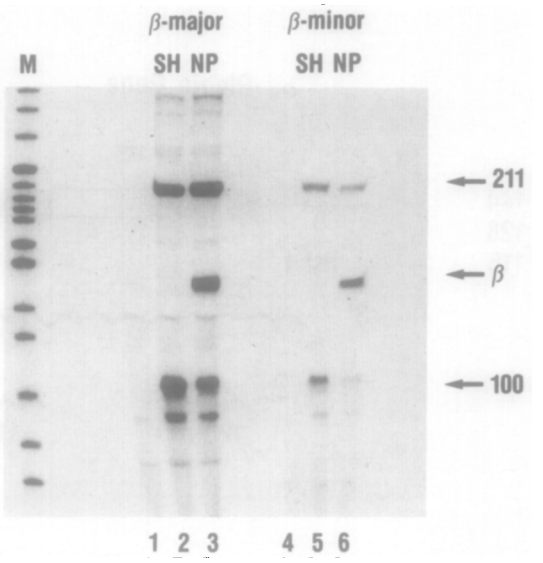

Figure 4. RNA analysis of transfected βmaj- and βmin-globin genes. Cells were transfected with 10μg DNA. βmajpBR327 (lane 1), βmajpBR327SH (lane 2), βmajpBR327NP (lane 3), βminpBR327 (lane 4), βminpBR327SH (lane 5), or βminpBR327NP (lane 6). Each lane was loaded with 20μg RNA. RNase digestion products were analyzed by electrophoresis on an 8% polyacrylamide -8M urea gel.

We first investigated the generation of mRNA by globin genes with neither ori nor enhancer sequences. A distinct low level of α_1 mRNA was detected in COS cells transfected with α_1pBR327 (Fig. 3, lane 1), whereas neither βmaj nor βmin transcripts were detected in βmajpBR327 or βminpBR327 transfected cells (Fig. 4, lanes 1 and 4). In transfectants with the replication competent plasmid, α_1pBR327SH, the amount of correctly initiated α_1 mRNA (Fig. 3, lane 2) was at least 100 fold that from the plasmid without the SV40 ori. In addition we detected aberrant transcripts, initiated upstream of the α_1-globin gene cap site. Both βmajpBR327SH and βminpBR327SH generated no normal transcripts, only aberrant, upstream transcripts (Fig. 4, lane 2 and 5). The 211nt βmaj fragment is the homolog of the full length probe, while the 90nt fragment reflects 3' cryptic splice sites within the βmaj first exon (6,7,23,24).

The third set of plasmids (NP series) examined the response of globin genes to an exogenous enhancer. Transfection with α_1pBR327NP generated roughly 30-fold the α_1 mRNA transcripts generated by the nonreplicating, enhancerless plasmid (Fig. 3, lanes 1 and 3). Both βmajpBR327NP and βminpBR327NP also generated proper transcripts (Fig. 4, lanes 3 and 6), at a βmaj:βmin ratio of roughly 4:1. This ratio was not due to differences in transfection

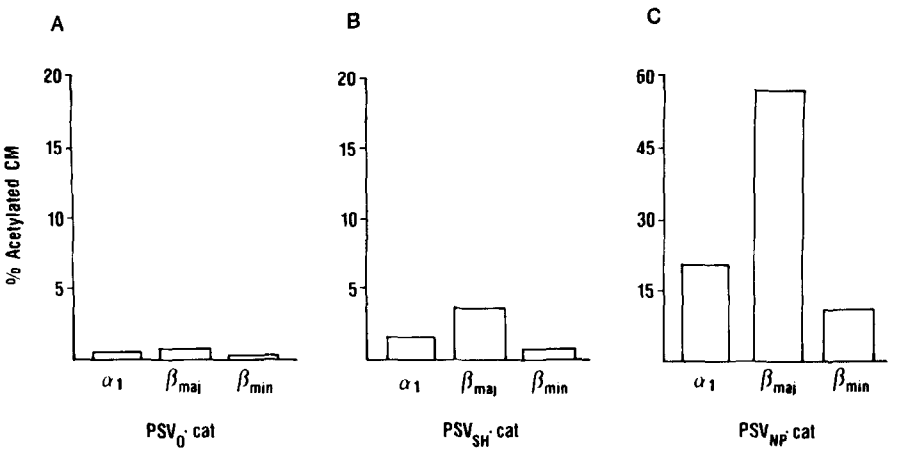

Figure 5. Expression of CAT at 48 hrs in COS cells transfected with 10μg globin(GRS)-CAT hybrids. Thin layer chromatography separates chloramphenicol (CM) from its acetylated forms. Spots were excised from the gels, quantitated by scintillation counting, and converted to percent CM acetylated. Each bar represents the average from three experiments.

efficiency or RNA recovery, since a cotransfected α_1 reference plasmid (data not shown) generated equivalent α mRNA in both transfectants.

Globin promoter-directed CAT gene expression in COS cells

To determine whether sequences located within the promoters of the mouse globin genes may be responsible for the differential transcription of intact globin genes, we assayed CAT gene expression from hybrid genes directed by globin promoter sequences. Cells transfected with α_1, βmaj, or βminPSV_0-cat plasmids, which contain neither SV40 ori nor enhancer sequences, express insignificant levels of CAT activity (Fig. 5A). SV40 ori (PSV_{SH}-cat series) only modestly increases CAT expression (Fig. 5B). CAT activity from all three globin-CAT plasmids with the SV40 enhancer (NP series) was substantial (Fig. 5C) and differential expression was unaffected. βmajPSV_{NP}-cat produced the most activity (56% chloramphenicol conversion), while $\alpha_1 PSV_{NP}$-cat and βminPSV_{NP}-cat converted 20% and 13.0%, respectively. Since there are two active mouse adult α-globin genes (α_1 and α_2), it is not surprising that the α_1 promoter is less active than the βmaj promoter for it represents half the potential α promoter activity found in vivo.

To insure that enhancer-dependent CAT transcription is directed exclusively by the globin regulatory sequences, we transfected a control construct containing the βmin-globin 5' sequence in an inverted orientation (25). This construct generates

no increased CAT activity (data not shown). It is not excluded, of course, that aberrant initiations and differences in RNA stability may also affect the relative CAT activity generated from these genes. Nevertheless, taken together, the observations on intact globin gene expression and on the 5' flanking regions suggest that differential expression of the three globin genes in COS cells and, by inference, in mouse erythroid cells, is determined by sequences located 5' to the coding regions. The level of α_1-globin gene expression appears dependent, in part, upon two activities, one located 3' to the cap site, which provides enhancer-like activity (26), and another in the 5' regulatory region, which influences the relative level of expression compared to the β-globin genes.

Acknowledgements

These studies were supported, in part, by grants from the National Cancer Institute (CA-31768 and CA-08748) and the Bristol-Myers Cancer Research Award.

References

1. Marks, P.A., and Rifkind, R.A. (1978) Ann. Rev. Biochem. 47, 419-448.
2. Nudel, U., Salmon, J., Fibach, E., Terada, M., Rifkind, R.A., Marks, P.A. and Bank, A. (1977) Cell 12, 463-469.
3. Alter, B.P., and Goff, S.C. (1978) Blood 52, 1047-1057.
4. Mellon, P., Parker, V., Gluzman, Y. and Maniatis, T. (1981) Cell 27, 279-288.
5. Humphries, R.K., Ley, T., Turner, P., Moulton, A.D. and Nienhuis, A.W. (1981) Cell 30, 173-183.
6. Triesman, R., Green, M.R. and Maniatis, T. (1983) Proc. Natl. Acad. Sci. USA 80, 7428-7432.
7. Konkel, D.A., Maizel, J.V., Jr. and Leder, P. (1979) Cell 18, 865-873.
8. Gorman, C.M., Moffat, L.F. and Howard, B.H. (1982) Mol. Cell. Biol. 2, 1044-1051.
9. Birnboim, H.C., and Doly, J. (1979) Nucleic Acids Res. 7, 1513-1523.
10. Radloff, R., Bauer, W. and Vinograd, J. (1967) Proc. Natl. Acad. Sci. USA 57, 1514-1521.
11. Soberon, X., Covarrubias, L. and Bolivar, F. (1980) Gene 9, 287-305.
12. Nishioka, Y., and Leder, P. (1979) Cell 18, 875-882.
13. Leder, P., Hansen, J.N., Konkel, D., Leder, A., Nishioka, Y. and Talkington, K. (1980) Science 209, 1336-1342.
14. Loyter, A., Scangos, G.A. and Ruddle, F.H. (1982) Proc. Natl. Acad. Sci. USA 79, 422-426.
15. Glisin, V., Crkvenjakov, R. and Byus, C. (1974) Biochemistry 13, 2633-2637.
16. Ullrich, A., Shine, J., Chirgwin, J., Pictect, R., Tischer, E., Rutter, W.J. and Goodman, H.M. (1977) Science 196, 1313-1318.

17. Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. and Green, M.R. Nucleic Acids Res. 12, 7035-7056.
18. Salditt-Georgieff, M., Sheffery, M., Krauter, K., Darnell, J.E., Jr., Rifkind, R.A. and Marks, P.A. (1984) J. Mol. Biol. 172, 437-450.
19. Sheffery, M., Marks, P.A. and Rifkind, R.A. (1984) J. Mol. Biol. 172, 417- 436.
20. Gluzman, Y. (1981) Cell 23, 175-182.
21. Banerji, J., Rusconi, S. and Schaffner, W. (1981) Cell 27, 299-308.
22. Moreau, P., Hen, R., Wasylyk, B., Everett, R., Gaub, M.P. and Chambon, P. (1981) Nucleic Acids Res. 9, 6047-6068.
23. Grosveld, G.C., deBoer, E., Shewmaker, C.K. and Flavell, R.A. (1982) Nature 295, 120-126.
24. Dierks, P., van Ooyen, A., Cochran, M.D., Dobkin, C., Reiser, J. and Weissman, C. (1983) Cell 32, 695-706.
25. Deschamps, J., Meijlink, F. and Verma, I.M. (1985) Science 230, 1174-1177.
26. Charnay, P., Treisman, R., Mellon, P., Chao, M., Axel, R. and Maniatis, T. (1984) Cell 38, 251-263.