Retroviral-mediated Transfer and Expression of Human β-Globin Genes in Cultured Murine and Human Erythroid Cells*

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We have cloned human β-globin DNA sequences from a genomic library prepared from DNA isolated from the human leukemia cell line K562 and have used the retroviral vector pZip-NosSV(X)1 to introduce a 3.0-kilobase segment encompassing the globin gene into mouse erythroleukemia cells. Whereas the endogenous K562 β-globin gene is repressed in K562 cells, when introduced into mouse erythroleukemia cells by retroviral-mediated gene transfer, the β-globin gene from K562 cells was transcribed and induced 5-20-fold after treatment of the cells with dimethyl sulfoxide. The transcripts were correctly initiated, and expression and regulation of the K562 gene were identical to the expression of a normal human β-globin gene transferred into mouse erythroleukemia cells in the same way. We have also introduced the normal human β-globin gene into K562 cells using the same retrovirus vector. SP6 analysis of the RNA isolated from the transduced cells showed that the normal β-globin gene was transcribed at a moderately high level, before or after treatment with hemin. Based on these data, we suggest that the lack of expression of the endogenous β-globin gene in K562 cells does not result from an alteration in the gene itself and may result from a lack of factor(s) necessary for β-globin gene transcription. Retroviral-mediated transfer of the human β-globin gene may, however, uniquely influence expression of the gene in K562 cells.

The human globin genes are differentially expressed during development. ε- and δ-globin genes are expressed during the early embryonic period, followed by the expression of γ-globin later in fetal life, and then the expression of β-globin around the time of birth (1). In adults, α- and β-globin genes are expressed at high levels. In the K562 cell line (2), which was isolated from an adult patient with chronic myelogenous leukemia, only embryonic ε- and fetal γ-globin genes are expressed; the gene coding for β-globin is not expressed, even after induction by hemin (3, 4). The β-globin gene does not appear to be transcribed (5), yet restriction mapping of the β-globin gene indicates that no major insertions, deletions, or rearrangements in or adjacent to the gene have occurred (6, 7).

The expression of γ- but not of β-globin genes in K562 cells may reflect the stage of differentiation at which these cells are arrested, and therefore the cells may be useful for studying the mechanism(s) controlling the sequential activation of different globin genes. As a first step in such studies, we have asked in this report whether the β-globin gene isolated from K562 cells can be transcribed and regulated after stable integration in the DNA of different erythroid cells. A 3.0-kb fragment of the gene including 800 bp of 5' flanking sequences and 500 bp of 3' flanking sequences has been introduced into a retroviral vector, and virus produced from this vector was used to infect mouse erythroleukemia cells (MEL)1 and to generate stable transduced clones. We also report the use of the same retroviral vector to introduce a normal human β-globin gene into K562 cells in order to study the expression and transcriptional regulation of that gene.

**EXPERIMENTAL PROCEDURES**

Cloning of the K562 β-Globin Gene—High molecular weight DNA isolated from the K562 cell line was partially digested by MboI and was size purified by sucrose gradient ultracentrifugation. Two μg of DNA (17-22 kb) was ligated to phage EMV3 arms, and recombinant phage DNA was packaged in vitro and amplified. A bacteriophage containing the β-globin gene was isolated from 800,000 clones by hybridization with a β-globin gene labeled with 32P in the second intron. The identity of the cloned β-globin gene was verified by restriction mapping with different enzymes (EcoRI, BglII, HindIII, BamHI) and hybridization against the same 32P-labeled β-globin intron or an established β-globin gene sequence (kindly provided by P. Charnay, EMBL, and T. Maniatis, Harvard).

Cell Culture, DNA Transfection, and Viral Infection—NIH 3T3, $2$, and $\psi$AM cells were grown in Dulbecco's modified Eagle's medium containing 10% calf serum. The D2 subclone of the 745-P24 MEL cell line (provided by D. Houman, Massachusetts Institute of Technology) was grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. The human leukemic cell line K562 (provided by D. Tuan, Massachusetts Institute of Technology) was grown in RPMI 1640 medium containing 10% fetal calf serum and 10 mM Heps. Induction of MEL cells was performed by addition of 1.8% dimethyl sulfoxide to the culture medium for 4 days, and the cells were maintained at densities of 2-5 × 10⁶ cells/ml. Induction of K562 cells was performed by culturing the cells for 5 days in media containing hemin at a concentration of 20-50 μM.

Transfections of $2$ and $\psi$AM cells were performed with 10 μg of plasmid DNA by the calcium-phosphate technique of Graham and Van Der Erb as modified by Parker and Stark (8).

1 The abbreviations used are: MEL, mouse erythroleukemia; MsSO, dimethyl sulfoxide; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; kb, kilobase; bp, base pair.
Viral stocks were prepared by adding fresh Dulbecco's modified Eagle's medium containing 10% calf serum to a near confluent monolayer of cells and, after 18 h, filtering the supernatant through a 0.45 μm filter (Acrodisc, Gelman Sciences, Ann Arbor, MI). Infections of 3T3 cells were performed by incubating 5 × 10⁶ cells with 2 ml of virus in the presence of 8 μg of Polybrene (Aldrich) for 2.5 h. Eight ml of medium was added, and the cells were grown to confluence before being divided and added to selective medium (G418 at 0.5 mg/ml). MEL cells were infected by cocultivating 5 × 10⁶ cells, previously treated for 18 h with tunicamycin (0.2 μg/ml), with near confluent (5 × 10⁶ cells) virus-producing φ2 cells for 48 h. Infection of K562 cells was performed similarly but without previous treatment of the cells with tunicamycin; φAM producer cells were used for cocultivation. After infection, the nonadherent MEL or K562 cells were harvested, plated several times to remove residual φ2 or φAM cells, and then grown in nonselective medium. Infected cells were selected by growth in 1 mg/ml G418 for 10-14 days (MEL cells) or for 20 days (K562 cells). Clones were then isolated by plating cells in individual microtiter wells.

Total cellular RNA was prepared using a guanidine thiocyanate procedure (9). Southern and northern hybridization analyses were performed as described (10).

Synthesis of Complementary SP6 Probes and RNA Mapping—The SP6 plasmids used for synthesis of probes complementary to mouse and human β-globin genes in mRNA were kindly provided by P. Chambon and T. Maniatis. Transcription and hybridization of the probe to cellular RNA were performed as described (11). Briefly, the mouse and human β-globin SP6 plasmids were linearized with EcoRI prior to transcription. Transcription was performed using [γ-32P]UTP (2000 Ci/mmol), and 2-5 × 10⁶ cpm of probe was used for hybridization with each 10 μg of total cellular RNA. Hybridizations were carried out at 30°C for 16 h and, after digestion of free RNA with RNase T1 and RNase A, the hybrids were analyzed by electrophoresis under denaturing conditions on a sodium dodecyl sulfate, 6% polyacrylamide gel.

RESULTS

Introduction of the K562 and Normal β-Globin Genes into Retroviral Vectors—The genomic sequence isolated from the K562 library was 17 kb long, and extended from 30 bp upstream of the β-globin gene to 7 kb downstream of the β-globin gene. A 3.0-kb fragment, with 850 bp of 5' flanking sequences and 550 bp of 3' flanking sequences, was obtained by digesting the clone with HpaI and PstI. This fragment was inserted at the BamHI site of the vector pZip-NeoSV(X), after addition of BclI linkers. The orientation of the globin gene with respect to the upstream long terminal repeat of SV(X) was determined by digestion with EcoRI. LTR, MoMuLV long terminal repeat; φ, MoMuLV packaging sequence; 3' SS and 3' SS, MoMuLV splice donor and acceptor sequence; NEO, Nt5 neomycin resistance gene; SVori, simian virus 40 origin of replication; pbrori, pBR322 origin of replication.

Generation of Recombinant Viruses Encoding β-Encoding DNA Sequences—In order to generate transmissible virus encoding the K562 and normal β-globin genes, the respective plasmids were introduced into φ2 (14) and φAM (15) cells by DNA transfection, and the transfected cells were selected in G418 (16). φ2 cells are NIH 3T3 cells that have been stably transfected with murine leukemia virus proviral sequences deleted in “packaging” sequences required for the encapsidation of viral RNA into virions (14). After transfection of these cells with recombinant constructs, only the recombinant RNA genomes can be encapsidated and give rise to transmissible virus (12). φAM is a φ2-like packaging cell line (15) which provides for the generation of transmissible virus with amphotropic host range. Amphotropic virus is able to infect both murine cells as well as a wide variety of cell lines from other species, including human (17).

Among the G418-resistant clones, those producing high titers of recombinant virus were identified by assaying the virus in culture supernatants for the ability to confer G418 resistance in infected 3T3 cells. The titers of the φ2 clones ranged from 5 × 10⁸ to 10⁹ G418-resistant colony forming units/ml, while the titers of φAM clones ranged from 10⁷ to 10⁸ G418-resistant colony forming units/ml.

Infection of MEL Cells with Recombinant Virus Containing the K562 β-Globin Gene—Infection of MEL cells was achieved by cocultivating tunicamycin-treated (18) cells with the virus-producing φ2 cells for 48 h. After selection in G418-containing medium for 12 days, both populations and clones of G418-resistant MEL cells were isolated. To demonstrate that the recombinant retroviral genomes that carry the K562 β-globin genes were transferred intact to cells, DNA was isolated from selected G418 clones and analyzed by the method of Southern (19). As can be seen in Fig. 2A, in the case of each G418' isolate, the integrated provirus possessed a structure identical to that of the plasmid DNA used to generate the φ2 producers.
Moreover, cleavage with BamHI indicated that, in each case, either a single copy or two copies of proviral sequences were found integrated in the cellular DNA of the clones (Fig. 2B). Most likely, those tracks showing two hybridizing bands of unequal molarity (e.g. 3-3, 4-2) represent a mixture of two independently infected clones.

To examine the expression of the K562 β-globin gene sequences, total cellular RNA was isolated from selected clones either before or after dimethyl sulfoxide induction, and was analyzed by the SP6 RNA protection method (20) (Fig. 3). The human β-globin RNA probe used for these studies (13) protects a 351-nucleotide fragment extending from the mRNA cap site to the BamHI site in exon 2. A mouse β-globin probe was also used as a control for the dimethyl sulfoxide induction and to quantitate the absolute levels of human β-globin gene transcription. In the case of each G418 clone analyzed, the introduced K562 β-globin gene was transcribed in an inducible fashion. Moreover, the globin transcript was initiated correctly, as evidenced by comparison of the length of the protected probe fragment to that obtained with authentic human β-globin RNA (data not shown). The intensity of the signal representing the protected fragments provides a means of quantitating the abundance of the globin mRNA from K562 cells, since the RNA probe is present in excess. By comparison with the levels of mouse β-globin mRNA found in induced MEL cells, we estimate that transcription from the human β-globin gene is about one-hundredth the transcription from the endogenous mouse β-globin gene. Nevertheless, the level of β-globin mRNA in K562 cells is comparable to the level of normal human β globin mRNA found in MEL transformants harboring the normal human β-globin gene (data not shown).

Infection of K562 Cells with Recombinant Virus Containing the Normal Human β-Globin Gene—To introduce the normal β-globin gene into K562 cells, the cells were cocultivated with

![Fig. 3. Induction of human β-globin mRNA in MEL cell clones infected with the K562 β-globin gene. MEL cell clones were induced by 5 days growth in medium containing 1.8% Me2SO. Total cellular RNA (10 μg) from uninduced (− lanes) or induced († lanes) cells was hybridized to an excess of 32P-labeled RNA probe complementary to a portion of the human β-globin mRNA, synthesized using the SP6 polymerization reaction. The protected fragment was separated by electrophoresis on a sodium dodecyl sulfate, 6% polyacrylamide gel. The schema indicates the expected protected fragment resulting from the SP6 hybridization analyses.](image)

![Fig. 4. Expression of the normal human β-globin gene in K562 cells. Populations of infected K562 cells were induced by 5 days growth in medium containing 50 mM hemin. Total cellular RNA (10 μg) from uninduced (− lane) or induced († lane) cells was hybridized to an excess of 32P-labeled RNA probe complementary to a portion of the human β-globin mRNA, synthesized using the SP6 polymerization reaction. Controls are: MEL RNA from infected and induced MEL cells; C:RNA from uninfected and induced K562 cells.](image)

**DISCUSSION**

The experiments described in this report were designed to determine the basis for the lack of β-globin gene expression observed in the human leukemic cell line, K562. Previous reports had shown that the β-globin gene was inactive transcriptionally, before or after hemin induction, yet no recognizable lesion in the gene could be detected (6). While several groups reported the inactivity of the K562 β-globin gene in transient expression assays utilizing cos and HeLa cells (21, 22), we wished to verify the integrity of the gene by stably introducing the sequences into erythroid cells. Since we had previously shown expression of the normal human β-globin gene after its transfer into MEL cells using retroviral vectors (13), similar constructs which incorporated the K562 β-globin gene were made and used to generate transmissible virus. The results presented here confirm those obtained in the transient expression assays and strongly argue against any structural mutation being responsible for the lack of expression of the endogenous β-globin gene in K562 cells. It may be important, however, that the DNA segment containing the β-globin gene used in our studies comprised only 815 bp of 5' sequence flanking the gene and 550 bp of 3' flanking sequence. It is possible that structural mutations exist in sequences not included in our constructions.

In a second series of experiments, the normal human β-globin gene was introduced into K562 cells to determine whether an exogenously introduced β-globin gene could be expressed in those cells. In two previous reports, Young et al. (23) and Kioussis et al. (24) showed that DNA fragments encompassing the human β-globin gene were not efficiently expressed after their stable introduction into K562 cells by DNA transfection. More recent studies by Grosveld and co-workers (25) show that the β-globin transcription seen in a minority of Puttko cell transformants (Puttko cells are derived from K562 cells) was due to aberrantly initiated β-globin gene
transcripts. Our results are in contrast to those findings and raise questions about the factors that might account for the discrepant results. An obvious difference between the experiments resides in the different methods of gene transfer employed. It might be argued, for example, that the integration of the β-globin gene sequences within the context of a transcriptionally active provirus could account for the activity of inserted β-globin gene sequences. While this may be true, it is unlikely for several reasons. First, in the studies of Cone et al. (13), the β-globin gene sequences present in the SV(X) vector were shown to be transcriptionally inactive after transfection. It might be argued, for example, that the integration of the 8-globin gene sequences within the context of a retrovirus could raise questions about the factors that might account for the expression of the 8-globin gene in an inappropriate developmental context. In addition, the use of viral vectors lacking the retroviral enhancer sequences indicated that the transcription of human β-globin gene sequences in erythroid cells was independent of retroviral transcription in these cells (13). Nevertheless, it is possible that in a case in which some but not all of the transacting factors necessary for β-globin gene transcription are available (which may be the case in K562 cells), the viral enhancer sequences may play some role in activating β-globin gene transcription. For this reason, it will be important to test the enhancer-deficient β-globin gene recombinants directly for transcriptional activity in K562 cells. Even if effects of viral enhancer are found, our results still suggest that at least part of the transcriptional machinery necessary for β-globin gene expression is present in K562 cells. Alternatively, it is possible that the chromosomal sites at which retroviruses are integrated may differ from those involved in DNA transfection and may be more accessible to tissue-specific transcription factors.

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