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Genetics

Early myeloid cell-specific expression of the human cathepsin G gene in transgenic mice

(hematopoiesis/serine proteases/gene regulation)

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ABSTRACT **The human cathepsin G (CG) gene is expressed only in promyelocytes and encodes a neutral serine protease that is packaged in the azurophil (primary) granules of myeloid cells. To define the cis-acting DNA elements that are responsible for promyelocyte-specific "targeting," we injected a 6-kb transgene containing the entire human CG gene, including coding sequences contained in a 2.7-kb region, ≈2.5 kb of 5′ flanking sequence, and ≈0.8 kb of 3′ flanking sequence. Seven of seven "transient transgenic" murine embryos revealed human CG expression in the fetal livers at embryonic day 15. Stable transgenic founder lines were created with the same 6-kb fragment; four of five founder lines expressed human CG in the bone marrow. The level of human CG expression was relatively low per gene copy when compared with the endogenous murine CG gene, and expression was integration-site dependent; however, the level of gene expression correlated roughly with gene copy number. The human CG transgene and the endogenous murine CG gene were coordinately expressed in the bone marrow and the spleen. Immunohistochemical analysis of transgenic bone marrow revealed that the human CG protein was expressed exclusively in myeloid cells. Expression of human CG protein was highest in myeloid precursors and declined in mature myeloid cells. These data suggest that the human CG gene was appropriately targeted and developmentally regulated, demonstrating that the cis-acting DNA sequences required for the early myeloid cell-specific expression of human CG are present in this small genomic fragment.**

Several genes encoding neutral serine proteases are expressed exclusively in immature myeloid precursors at the promyelocyte/promonocyte stage of development (1–3). Expression of these proteases is concomitant with the production of the azurophil (primary) granules in which these proteases are packaged (4–6). Recently, neutrophil elastase and two highly related serine protease genes (proteinase 3 and azurocidin) have been shown to be clustered in a single genetic locus on human chromosome 19 (3). These three genes are coordinately expressed during the promyelocyte stage of myeloid differentiation (1, 3), suggesting that a unique regulatory mechanism controls expression of all of the genes in this locus.

Like expression of the other azurophil granule-associated serine proteases, expression of cathepsin G (CG) is restricted to the promyelocyte/promonocyte stage of development (2, 7). Interestingly, the CG gene and three other CG-like genes, encoding human granzyme B (CGL-1), human granzyme H (CGL-2), and mast cell chymase, form a cluster of hematopoietic serine protease genes located on chromosome 14 (8, 9). Unlike the elastase-like genes on chromosome 19, the genes in this cluster are each expressed in distinct hematopoietic lineages (8). The fact that each of the genes in this cluster is expressed in a distinct hematopoietic lineage suggests that separate mechanisms regulate the expression of each gene. Nevertheless, the restricted expression of CG in promyelocytes makes it an excellent model to study the factors that control myeloid development.

To identify the cis-acting regulatory sequences controlling the lineage- and development-specific expression of CG, we have examined the pattern of expression of a 6-kb human CG genomic fragment in transgenic mice. We have found that this fragment is capable of directing the restricted expression of human CG to cells at an early stage of myeloid development.

MATERIALS AND METHODS

Production of Transgenic Mice. The previously described 6-kb CG genomic fragment (10) was purified following release from pUC9 by digestion with *Sal* I and *Bam*HI. Transgene microinjections and generation of transgenic mice were performed as described (11). For transient transgenic studies, embryos were harvested at day 15 of gestation (e15), at which time a hind leg was removed for the purpose of extracting DNA. Individual embryos were then surrounded and frozen in tissue embedding medium (Histoprep; Fisher Scientific) and stored at −70°C.

Identification of Transgene-Positive Embryos and Mice. Genomic DNA purified from embryo legs or from tail segments was analyzed by Southern blotting (12). Blots were probed with a unique random-primer-labeled 250-bp *Xba* I–*Hin*dIII fragment derived from the fifth exon and 3′ flanking region of the human CG gene. These blots were reprobed with a unique 250-bp fragment (generated by PCR amplification) derived from the 5′ end of the murine granzyme A gene to provide a control for gene copy number. Comparison of densitometric scans of the signals corresponding to these two probes was used to estimate transgene copy number.

RNA Purification and S1 Nuclease Analysis. Total cellular RNA was prepared as described (11). End-labeled probes were prepared and S1 nuclease protection assays were performed (2, 7).

Immunohistochemistry and Immunofluorescence. Five-micrometer-thick midsagittal cryostat sections of seven transgene-positive and two transgene-negative embryos were prepared for analysis. Slides of transgene-positive and -negative bone marrow cells were prepared by centrifugation for 5 min at 500 rpm using the Cytospin 3 cell-preparation system (Shandon, Pittsburgh). Primary antibodies [affinity-purified guinea pig anti-human CG antiserum (generously provided by L. Heck; ref. 13) and/or a rat anti-mouse monoclonal antibody (7/4, Serotec)] diluted in phosphate-buffered saline were applied at room temperature for 1 hr. For immunohis-

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Abbreviations: CG, cathepsin G; e15, embryonic day 15.

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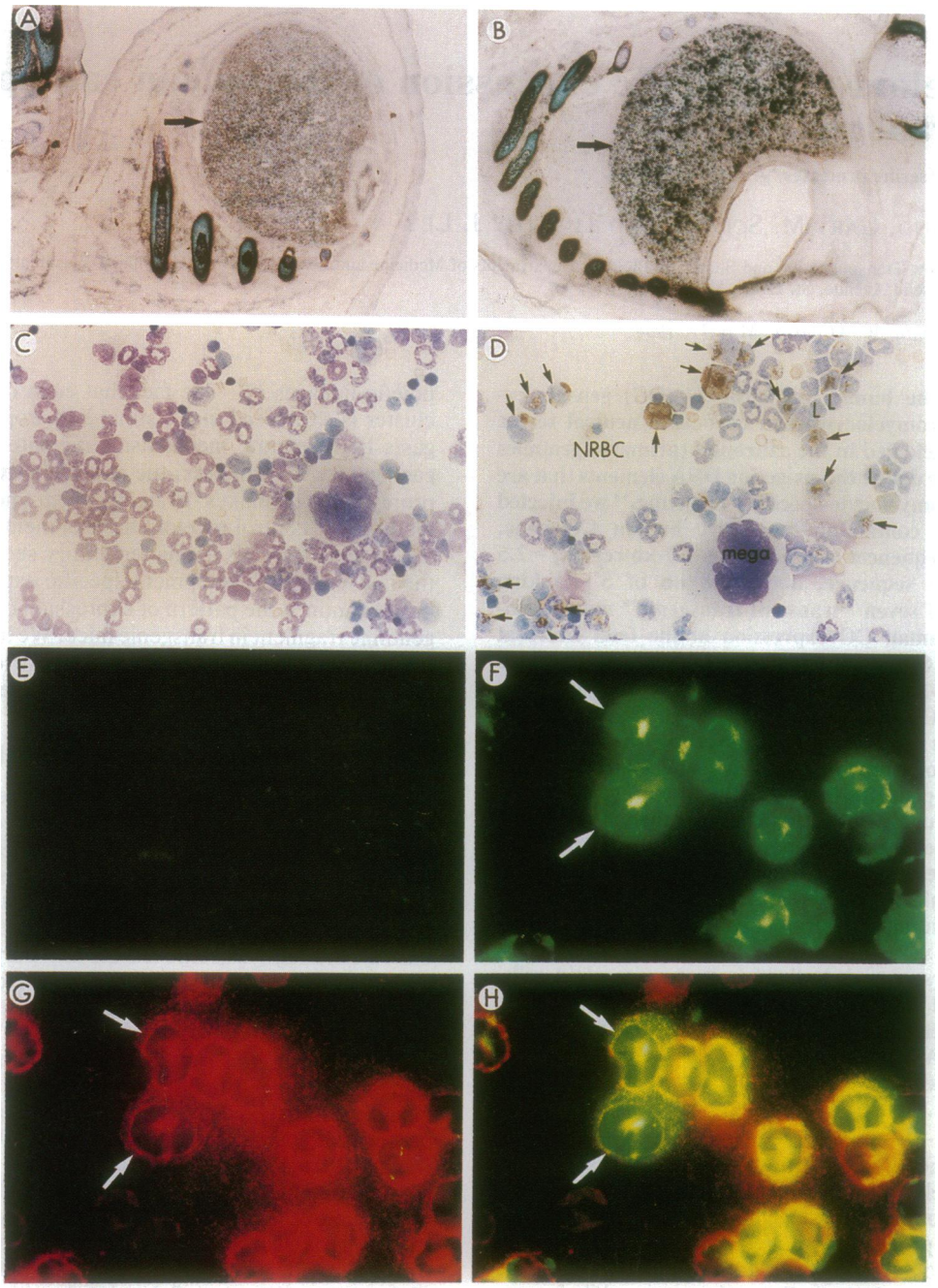

FIG. 1. The human CG transgene is expressed exclusively in the myeloid cells of transgenic bone marrows. (*A* and *B*) Immunohistochemical studies with human CG transgene-negative (*A*) and transgene-positive (*B*) sectioned e15 embryos, using the affinity-purified anti-human CG antibodies. In *A*, a transgene-negative embryo reveals nonspecific background staining that was also present in the absence of primary antibody. No specific staining is seen in the fetal liver (designated by the arrow). In *B*, an embryo with 12 copies of the human CG transgene contains discrete cells in the fetal liver stain that are positive for human CG protein. No other organs or tissues demonstrated significant staining with the anti-human CG antibodies. The intensity of staining observed in the fetal liver of this embryo was indistinguishable from that of embryos containing 17 or 21 copies of the transgene. (*C* and *D*) Bone marrow from a transgene-negative littermate (*C*) and a transgene positive mouse from founder line 12 (*D*) analyzed with immunohistochemical staining using the anti-human CG antibody. No brown/black precipitate is detected within any cell in *C*. In *D*, however, the precipitate is found exclusively in cells of myeloid morphology; most of the intensely staining cells are marked with arrows. Nucleated red cells (NRBC), lymphoid cells (L), and a megakaryocyte (mega) reveal no specific staining. (*E–H*) Colocalization of human CG and a mouse myeloid marker. In *E*, bone marrow from a nontransgenic littermate was stained with the anti-human CG antiserum. In *F–H*, transgenic bone marrow from founder line 17 was stained with anti-human CG antibodies and with the rat monoclonal antibody 7/4 (directed against a murine myeloid-specific antigen). In *H*, a double exposure of the field in *F* and *G* is shown. Note that essentially all of the cells staining with anti-human CG antibodies (*F*) and the murine 7/4 antibody (*G*) have a myeloid nuclear morphology. In *H*, the double exposure of *F* and *G* reveals yellow staining of nearly all the positive cells, indicating colocalization of the human CG protein and the myeloid 7/4 protein. The two cells designated by the arrows in *F–H* indicate immature myeloid precursors where colocalization of the two antigens clearly occurs, but where green staining is significantly greater than red. This suggests that the human CG protein is expressed at an earlier stage in myeloid differentiation than the protein detected by the 7/4 antibody.

tochemistry, slides were subsequently incubated for 30 min with goat anti-guinea pig IgG-biotin conjugate (Sigma) diluted 1:100 in phosphate-buffered saline. Then, following a tertiary incubation with streptavidin-alkaline phosphatase conjugate

(Sigma) at a 1:100 dilution, slides were stained with the alkaline phosphatase substrate kit II (Vector Laboratories). For immunofluorescence, slides were secondarily incubated in the dark for 45 min with goat anti-guinea pig IgG-fluorescein conjugate (Vector Laboratories) and goat $F(ab')_2$ anti-rat IgG-phycoerythrin conjugate (Tago), both diluted 1:500 in phosphate-buffered saline.

RESULTS

Human CG Transgene Is Expressed in Liver of e15 Transgenic Mouse Embryos. To identify cis-acting DNA sequences involved in the regulation of the CG gene, we isolated a 6-kb *Sal* I–*Bam*HI human genomic fragment encompassing the intact human CG gene (containing 5 exons and 4 introns; refs. 8 and 9) and examined its expression in transgenic mice. This fragment contains ≈2.5 kb of 5′ flanking sequence and ≈0.8 kb of 3′ flanking sequence; if the transcription initiation site is designated +1, the translation initiation site is located at +29 and the poly(A) addition site is at +2698 (8, 9). We used a previously described approach for rapid analysis of the expression of this construct in transgenic mice, which will subsequently be referred to as the "transient transgenic" assay (14); it relies on the fact that the fetal liver is the major site of hematopoiesis in a developing mouse embryo at e15.

Following microinjection of the human CG transgene, individual mouse embryos were harvested on e15 and frozen in embedding medium. Frozen sections of transgene-positive embryos were then assayed for expression of human CG protein by immunohistochemistry using a polyclonal affinity-purified guinea pig antiserum raised against human CG protein. Shown in Fig. 1*A* is a section of a transgene-negative embryo after immunohistochemical staining with the antiserum to human CG. No specific staining was detectable throughout this embryo (regions staining brown-black were shown to be present in the absence of the primary antibody). In particular, the fetal liver (indicated by the arrow) did not stain positive for human CG protein. When a transgene-positive embryo was analyzed (Fig. 3*B*), discrete populations of cells within the fetal liver were detected by the anti-human CG antiserum (brown-black in color). No other organs or tissues demonstrated specific staining. Seven transgene-positive embryos (with 1–21 copies of the transgene) were examined by immunohistochemistry; all revealed the same pattern of staining. Expression of human CG protein in the fetal livers of these embryos correlated roughly with transgene copy number (data not shown). The restricted expression of human CG protein to the fetal livers of these embryos is consistent with appropriate regulation of this transgene. These results provided a basis for establishing stable founder lines with this transgene to examine its expression in the hematopoietic cells of adult mice.

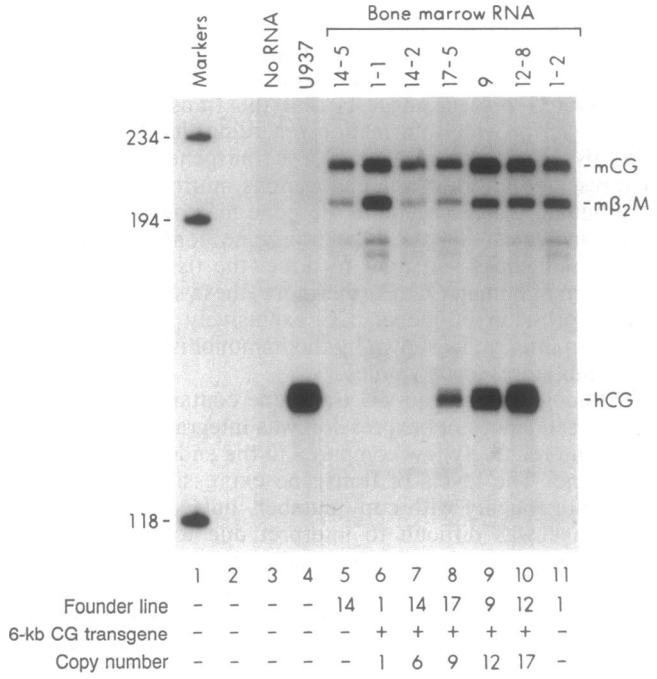

FIG. 2. Expression of the human CG transgene in the bone marrow of transgenic mice. Ten micrograms of total cellular RNA derived from each indicated source was hybridized simultaneously with probes specific for murine CG (mCG), murine β_2-microglobulin (mβ_2M), and human CG (hCG) mRNA. After hybridization, samples were treated with S1 nuclease as described (7) and then electrophoresed in an 8% polyacrylamide sequencing gel. Positions of probe fragments corresponding to correctly spliced murine CG, mβ_2M, and hCG mRNA are shown at right. Sizes (in nucleotides) of molecular markers derived from *Hae* III-cleaved ϕX174 phage DNA are shown at left. The source of each bone marrow RNA sample is shown at the bottom, as well as the presence or absence of the hCG transgene and the gene copy number per haploid genome. Note that each mouse bone marrow sample contains a relatively constant ratio of mCG to mβ_2M mRNA. In founder lines 1, 17, 9, and 12, there is a correlation between gene copy number and the level of hCG expression. U937 is a human promonocyte cell line.

Expression of the Human CG Transgene Is Detected in the Bone Marrow of Adult Transgenic Mice. To examine the expression pattern of the human CG transgene in adult mice, the 6-kb genomic fragment was reinjected. Five transgene-positive mice were identified by Southern blot analysis using DNA extracted from tail segments. Transgene-positive mice from all five founder lines were sacrificed for analysis of CG expression in bone marrow, the major hematopoietic organ in adult mice, by S1 nuclease protection assay (Fig. 2). RNA from the human promonocytic U937 cell line (lane 4) provided a positive control for human CG transcripts (8, 10). Bone marrow RNA from transgene-negative littermates (lanes 5 and 11) showed the protected bands for endogenous murine CG and β_2-microglobulin mRNAs; β_2-microglobulin provided a control for integrity of the RNA samples. Expression of the human CG transgene was detected in four of the founder lines by this assay and was integration-site dependent. For most lines, expression of the human CG transgene was relatively low per gene copy with respect to the endogenous murine CG gene (Table 1). However, despite the position effects of integration, expression of the human CG transgene did correlate roughly with copy number.

High-Level Expression of the Human CG Transgene Is Restricted to the Bone Marrow of Adult Mice. S1 nuclease protection assays using RNAs purified from the major organs of a mouse derived from founder line 12 showed high-level

Table 1. Copy-number-corrected expression of the human CG transgene in the bone marrow of transgenic mice

Transgenic founder line	Transgene copy number	Human CG/ murine CG expression*	Copy-number-corrected expression†
1	1	0.06	0.06
14	6	0.00	0.00
17	9	2.24	0.25
9	12	2.84	0.24
12	17	11.3	0.68

*The ratio of human CG mRNA to murine CG mRNA was quantitated by densitometric scanning of signals generated by S1 nuclease protection (see Fig. 2).

†The human CG/murine CG mRNA ratio divided by transgene copy number, determined by densitometric comparison of human CG transgene signals to granzyme A gene signals on Southern blot analysis (data not shown).

expression of the murine CG gene and the human CG transgene in the bone marrow (Fig. 3, lane 5), as expected. In addition, very small amounts of coordinately expressed murine CG and human CG transcripts were also present in the spleen (lane 10), an hematopoietic organ in young mice. Mice from line 17 had an identical pattern of transgene expression (data not shown).

Localization of Human CG Expression to Myeloid Precursor Cells in the Bone Marrow. We next wanted to determine whether expression of the human CG transgene was restricted to myeloid cell types in the bone marrow. In bone marrow from a transgene-negative littermate after staining with the antiserum specific for human CG protein, no expression of human CG protein was detected in any cell type (Fig. 1*C*). Transgene-positive bone marrow cells (founder line 12) showed intense cytoplasmic staining in cells with large, doughnut-shaped nuclei (Fig. 1*D*, arrows), probably early myeloid precursor cells. The staining was clearly less intense in the smaller, more mature myeloid cells, which may reflect appropriate down-regulation of the human CG transgene during myeloid maturation. No expression of human CG protein was observed in cells of the megakaryocytic, erythroid, or lymphoid lineages. Bone marrow from founder lines 9 and 17 had an identical staining pattern (data not shown).

To confirm accurate targeting of human CG transgene to the myeloid lineage by colocalizing human CG protein with a murine myeloid protein, we analyzed transgene-negative and -positive bone marrow cells by immunofluorescence with the anti-human CG antiserum and a monoclonal rat anti-mouse antibody, 7/4, that detects a murine myeloid-specific protein (14). The anti-human CG antiserum did not detect any cell type in transgene-negative bone marrow (Fig. 1*E*). However, distinct cells (green in appearance) in transgene-positive bone marrow from founder line 17 were detected by the anti-human CG antiserum (Fig. 1*F*). The 7/4 antibody detected the same cells (red in appearance, Fig. 1*G*). The colocalization of human CG protein with the neutrophil marker recognized by 7/4 produced a yellow signal in these cells upon dual exposure (Fig. 1*H*). Essentially all of the cells detected by the anti-human CG antiserum and the 7/4 antibody had a myeloid nuclear morphology. However, the larger, more immature myeloid precursors (arrows) appeared more green than yellow (Fig. 1*H*), indicating a higher level of human CG transgene expression in those cells. We were also able to detect the 7/4 antigen in human CG-expressing cells within the fetal livers of transgene-positive embryos (data not shown). Finally, simultaneous staining with the anti-human CG antibody and monoclonal antibodies directed against murine macrophage markers (F4/80 and MOMA-2, BioSource International, Camarillo, CA) revealed no evidence for colocalization (data not shown). These results strongly suggest that expression of the human CG transgene was correctly targeted to the early myeloid cells in the bone marrow of adult mice and e15 livers.

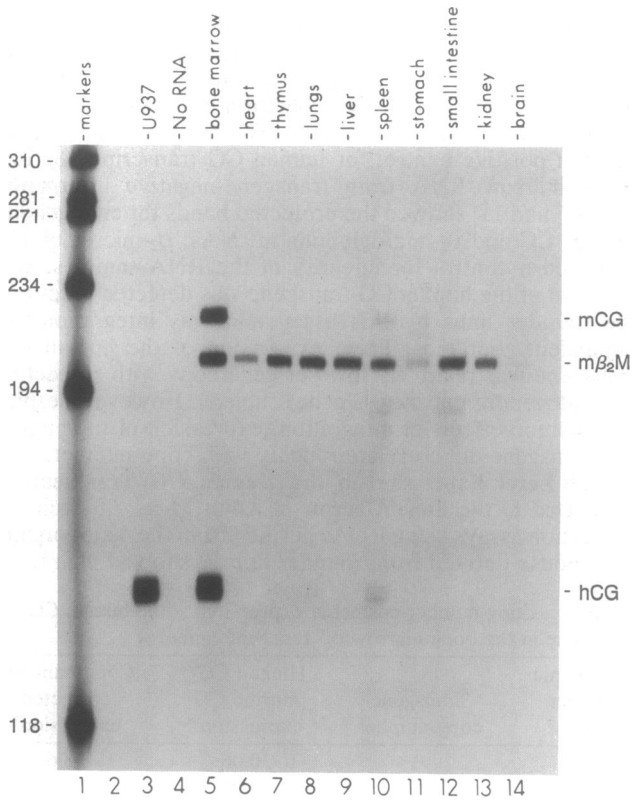

FIG. 3. The human CG transgene and the endogenous murine CG gene are expressed only in the bone marrow and spleen. An S1 nuclease protection analysis identical to that described in Fig. 2 is presented. Ten micrograms of total cellular RNA derived from the indicated organs of a transgenic F_1 animal from founder line 12 was hybridized simultaneously with probes for murine CG (mCG), murine β_2-microglobulin (mβ_2M), and human CG (hCG) mRNA. Note that hCG and mCG are primarily expressed in the bone marrow, although smaller amounts of both mRNAs are present in the spleen (lane 10).

DISCUSSION

This study has demonstrated that a 6-kb human CG transgene is regulated appropriately in a lineage- and development-specific manner in transgenic mice. Colocalization of the neutrophil 7/4 antigen in fetal liver cells staining positive for human CG protein suggests that the transient transgenic assay can provide information regarding the development- and tissue-specific regulation of a transgene. The identical expression pattern of the endogenous murine CG gene and the human CG transgene in the bone marrow of transgenic mice indicates that the 6-kb transgene contains cis-acting DNA sequences sufficient to direct the tissue-specific expression of human CG. Furthermore, these sequences target the expression of human CG exclusively to cells of the myeloid lineage, as shown by the immunohistochemical and immunofluorescence studies.

Although the human CG transgene contains myeloid targeting sequences, its expression was integration-site dependent and relatively low compared to the endogenous murine CG gene. The levels of transgene expression appeared to correlate roughly with copy number, but copy-number dependence was difficult to interpret due to the effects of transgene integration. Interestingly, the 5′ flanking region from another serine protease gene in the cluster on chromosome 14, encoding granzyme B, can appropriately target the expression of a heterologous gene to activated cytotoxic T cells; however, this transgene is also strongly influenced by position effects of integration (10). Therefore, it is likely that the 6-kb human CG genomic fragment and the 5′ flanking sequence of the granzyme B gene lack regulatory information (e.g., locus control) that insulates each gene from the effects of surrounding chromatin (15, 16). A locus-control-like element may exist within or near the serine protease gene cluster that interacts with tissue-specific elements near each gene to permit high-level, lineage-specific expression. Alternatively, each gene within the cluster may have its own regulatory sequences that shield it from the effects of surrounding chromatin (17).

CG expression is normally restricted to the promyelocyte stage of myeloid development (2, 7). During this stage, CG protein is packaged into azurophil granules, which are distributed to the postmitotic, late-stage daughter cells as myeloid differentiation proceeds (4–6). Our immunohistochem-

ical and immunofluorescence data demonstrate that expression of human CG protein in the bone marrow isolated from transgenic mice was most abundant in cells having an early myeloid morphology and that expression declined in more differentiated cells. This pattern suggests that the human CG transgene was expressed only at an early stage of myelopoiesis (e.g., promyelocytes). The weaker intensity of staining in the more mature cells suggests that expression of the transgene was appropriately down-regulated and that these cells contained protein that was produced at an earlier stage of development. It is unlikely that this phenomenon was due to protein degradation, since human CG protein was also detected in peripheral blood leukocytes isolated from these lines of transgenic mice (data not shown). These data suggest that the 6-kb fragment contains the signals for promyelocyte targeting and for the down-regulation of the CG gene with terminal myeloid differentiation.

Genetic programs controlling the expression of promyelocyte-specific genes and the factors involved in this program are not well understood. Cis-acting DNA elements important for the regulation of the neutrophil elastase gene (18), the myeloperoxidase gene (19–21), and the promyelocyte-specific mim-1 gene (22) have been identified; however, the roles of these cis-acting elements for promyelocyte-specific targeting in transgenic animals have not been defined. Some information is available about transcription factors that bind to regulatory elements within or near these genes. For example, (*i*) an upstream element of the neutrophil elastase gene appears to be recognized by Ets family members (18), (*ii*) a regulatory element upstream from the myeloperoxidase promoter is recognized by the partially characterized MyNF-1 factor (21), and (*iii*) the mim-1 5′ flanking region contains binding sites for Myb, Ets, and C/EBP transcription factors (22–24). Although the 5′ flanking regions for human and mouse CG genes contain several conserved elements (7, 10), the functional significance of these elements, and the proteins that bind them, have not been characterized in detail.

Based on the information obtained in this study, we suggest that transient transgenic analysis will be useful for defining the cis-acting DNA elements required for myeloid-specific expression. Since this assay does not require the generation of founder lines, it can be more rapidly performed than standard transgenic studies; when coupled with colocalization studies, it can provide detailed information about the specificity of targeting.

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