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Genomic Organization of the Mouse Granzyme A Gene

TWO mRNAs ENCODE THE SAME MATURE GRANZYME A WITH DIFFERENT LEADER PEPTIDES*

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Granzyme A is a serine protease that, together with the other granular components of cytotoxic T lymphocyte (CTL) cells, has been implicated in the cytolysis process. We report here two different messages and the genomic organization of the mouse granzyme A gene. The granzyme A gene is composed of six exons spanning 7 kilobases. Alternative splicing of the second exon results in the two transcripts. The two mRNA species encode the same mature granzyme A protein but with different leader sequences. The first (HF1) encodes a typical leader signal sequence similar to other granzymes, but the second (HF2) putative leader sequence is different and less hydrophobic. Both messages are present in cultured CTL cell lines and in normal lymphoid tissues. They are both induced when CTL cells are activated *in vitro* or *in vivo*. Both messages can be translated *in vitro*, although the HF1 message appears to be much more efficient as a template. The putative 5′ promoter region of the HF gene sequenced (500 base pairs of upstream sequences) contains no well defined promoter sequences aside from the TATA box. The results suggest that (*a*) granzyme A may be produced with putative different leader sequences from two different mRNAs; (*b*) this may provide a model system for studying alternate splicing and the evolution of a complex enzymatic system in an organelle; and (*c*) the genomic DNA reported will be useful for studying transcription regulations involved in controlling the specific expression pattern of this gene.

Cytotoxic T lymphocytes (CTLs)[1] and natural killer cells may have cytoplasmic granules that are exocytosed upon binding to the target cells. These granules, which have been implicated in the killing process, contain perforin (cytolysin) (1, 2) and at least seven serine proteases called granzymes A through G (3). *In vivo*, the mRNAs for granzymes A and B are expressed in the cellular infiltrates in graft rejection (4) and viral infection (5). Granzyme A, also named Hanukah Factor (HF, Ref. 6), BLT esterase (7), TSP-1 (8), SE-1 (9), and CTLA-3 (10), is a neutral serine protease that cleaves after Arg or Lys residues. It also cleaves the colorimetric substrate benzyloxycarbonyl lysyl thioester (BLT). BLT cleavage activity has been shown to be released when CTLs are incubated with their target cells (11) or with specific anti-T cell receptor antibodies (12), or with some calcium ionophore (9).

Protease activity has long been implicated in the cell-mediated killing process (13–15). A potent inhibitor of BLT activity has been shown to inhibit the killing ability of a CTL (16). More recently, a CTL cell line whose granzyme A gene expression has been inhibited by an antisense gene shows reduced killing potential.[2] Using purified granzyme A, it has been shown that it can induce DNA degradation (18), a process associated with cell-mediated cytolysis (19).

Many serine proteases are synthesized with an amino-terminal peptide of 20–30 amino acids that is absent from the mature form. This leader peptide consists of a signal (pre-) peptide, which directs the nascent polypeptide into the lumen of the rough endoplasmic reticulum, and an activation (pro-) peptide, which keeps the protease in an inactive state (20). Granzyme A, like the other granzymes, does not have a leader peptide when it is isolated from the granules (21). It has been proposed that the acidic pH of the granule environment (8) and/or binding to the granule proteoglycans (22) keeps it inactive.

We have cloned the granzyme A gene and characterized its genomic organization. By analysis of its mRNA, we found that it expresses two different granzyme A mRNAs. They encode the same mature protein with two different leader peptides. Alternative splicing of the second exon of the granzyme A gene produces the two types of mRNA. One leader sequence is very similar to that of the other granular proteins such as perforin and the other granzymes. The other is different and less hydrophobic. Both messages are expressed in CTL lines and in normal lymphoid tissues, although the one with the typical granzyme leader sequence is much more abundant. Both messages are induced *in vivo* or *in vitro* during activation of CTL function.

MATERIALS AND METHODS

Isolation of Full-length cDNAs—A subclone of the HF cDNA (6) lacking the poly(A) tail was used to screen 10^5 recombinant bacteriophage each from the AR1 and 1E4 CTL cDNA libraries (6). Twenty positive clones were purified and their lengths determined by restriction mapping. The two longest clones were subcloned into M13mp18 or M13mp19 (23) for sequencing. The nucleotide sequence was deter-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L00631.

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[1] The abbreviations used are: CTL(s), cytotoxic lymphocyte(s); HF, Hanukah Factor; BLT, benzyloxycarbonyl lysyl thioester; kb, kilobase(s); PCR, polymerase chain reaction; bp, base pair(s).

[2] A. Talento, M. Nguyen, S. Law, J. K. Wu, M. Poe, J. T. Blake, M. Patel, T. J. Wu, C. Manyak, M. Silberklong, G. Mark, M. Springer, N. H. Sigal, I. Weissman, C. Bleackley, E. Podack, M. Tykocinski, and G. C. Koo, submitted for publication.

mined on both strands by the dideoxynucleotide chain termination method (24), and the Genepro program (Riverside Scientific) was used to analyze the sequence data and calculate sequence similarities.

Isolation of Genomic Clones—A murine genomic library in lEMBL3 (a kind gift of T. St. John, ICOS, Seattle, WA) was screened with the HF subclone described above. The 6×10^5 recombinant phages screened contained two classes of hybridizing inserts; the longest representative of each class was characterized further. Restriction mapping was done according to standard methods, and the digested DNA was transferred to Genetran (Plasco). The locations of the exon boundaries in the HF2 cDNA (described below) were approximated by comparison with the rat mast cell protease II gene (25), and restriction fragments corresponding to the putative exons were subcloned. These fragments, used as probes on the genomic clone blots, gave a rough map of the genomic organization. One of the genomic clones had a 13-kb insert that included the entire gene and 6 kb of upstream sequences. The other clone had 11 kb upstream and 4 kb of the gene, terminating between the third and fourth exons. Restriction fragments covering all of the exons and 1 kb upstream of the first exon were subcloned into M13 for sequencing. The sequences were determined on both strands as described above.

RNA Sources—RNA from the following cell lines was isolated in the presence of guanidium isothiocyanate and purified on CsCl gradients (26): AR1, an H-2^d-specific CTL line derived from the C57L mouse strain (6); 1E4, another C57L CTL line that kills Abelson virus-infected H-2^b targets (6); RAW112, an H-2^d expressing, Abelson virus-induced B cell lymphoma used as the AR1 target (6); and PC60, a cytolytic T cell hybridoma with inducible cytolytic activity by culturing for 3 days in Dulbecco's modified Eagle's medium with 25% supernatant from concanavalin A-stimulated rat spleen cells (27). (PC60 cells were kindly provided by M. Nabholz, Swiss Institute for Experimental Research, Epliages, Switzerland).

Primer Extension—Primer extension assay (28) was performed by end labeling 100 ng of an antisense oligonucleotide from the third exon (E3, sequence TACGGTCTTGAGTGAGGAAC) with 50 μCi of [γ-^{32}P]dATP (Amersham Corp.). One-tenth of this served as the primer for cDNA synthesis on RNA from AR1 (1 μg of poly(A)$^+$ and 5 μg of total RNA), C57BL/10 spleen and lymph nodes (15 μg of total RNA, kindly provided by C. Okada, Stanford University, CA), and RAW112 (15 μg of total RNA). The extension products were run on a 6% polyacrylamide gel, and HF2 sequencing reactions based on the above primer were used as size markers.

Skin Grafts—For the allogeneic skin grafts, tail skin pieces (20–30 mm^2) from male C57BL/Ka mice were grafted onto the left thorax of male BALB/c mice (29). Syngeneic grafts, BALB/c onto BALB/c and C57BL/Ka onto C57BL/Ka, and normal tail skin served as controls. Cells were isolated from the grafts after 8–10 days by a modification of the method for extracting dendritic epidermal cells (30). Briefly, the graft was removed, placed in 300 μl of GNK-trypsin (0.1% glucose, 14.8 mM NaCl, 5.3 mM KCl ,0.3% trypsin) and incubated at 8 °C overnight. After 16–20 h, the epidermal layer was pulled away from the dermis and incubated at 37 °C in 200 μl of GNK-trypsin, 100 μg/ml DNase A for 15 min with gentle shaking. The isolated cells were separated from the keratin debris, counted, spun through 10% bovine serum albumin, and frozen at −70 °C until analysis. Approximately 10^3–10^4 cells of assorted types were recovered from each graft.

Polymerase Chain Reaction Analysis—Poly(A)$^+$ RNA was isolated from the skin graft-infiltrated cells and from 10^6 RAW112 cells (negative control) according to the method of van de Rijn *et al.* (31). The mRNA was resuspended in 30 μl of 10 mM Tris, pH 7.5, 0.1 mM EDTA, and single-stranded cDNA was synthesized from 10 μl. From tissues and non-CTL lines, 1 μg of total RNA or 10 ng of poly(A)$^+$ RNA was used to make cDNA; from CTL lines, one-tenth that amount was used. One-third (10 μl) of the single-stranded cDNA was amplified in 25 (skin graft) or 22 (purified RNA) cycles of the polymerase chain reaction (32). For the skin grafts, an equal amount of every cDNA preparation was amplified with actin primers to ensure that mRNA had been recovered and that the cDNA synthesis had worked. The number of cycles was set by empirically determining the minimum amount of amplification that gave a visible HF2 band in a known positive sample. The reactions were done in a 100-μl volume with cycle times and temperatures of 30 s, 94 °C; 60 s, 50 °C; 60 s, 72 °C. The oligonucleotide primers had the following sequences: L1, CCATCTCTTGCTACTCTCCT (sense); L2, CAATGGAGATTGCTGCCGAT (sense); E5, TGCTACTCGGCATCTGGTTC, (antisense); actin 5′, CCACACCTTCTACAATGAGC (sense); and actin 3′, ATCTCCTTCTGCATCCTGTC (antisense). One-tenth of the PCR products was electrophoresed on a 1.5% agarose gel, transferred to Genetran (Plasco), and probed with an isolated fragment corresponding to exon 4.

In Vitro Transcription and Translation—The HF1 and HF2 cDNAs were cloned into pBluescript (Stratagene). The transcription and translation reactions were performed essentially as described (33), except that ^{14}C-labeled leucine and a rabbit reticulocyte system (Bethesda Research Laboratories) were used in the translation reaction. Equal amounts of RNA transcripts were used in the translation reactions. The translation products were examined by electrophoresis in a 12% polyacrylamide gel (30 acrylamide, 0.8 bisacrylamide) containing sodium dodecyl sulfate (34). A ^{14}C-methylated protein mixture (Bethesda Research Laboratories) was used as the molecular weight standard. After electrophoresis, the gel was fixed, treated with En^{3}Hance (Du Pont-New England Nuclear), and autoradiographed.

Transient Expression in COS-7 Cells—The HF1 and HF2 cDNAs were cloned into pSRa.SD2 (kindly provided by Dan Denney, Stanford, CA). The transfection procedure was essentially as described in the lipofectin (Bethesda Research Laboratories) information sheet. Briefly, 5 μg of the appropriate DNAs were mixed with 1.5 ml of Optimum (Bethesda Research Laboratories) medium and then mixed with 1.5 ml of the same medium containing 40 μg of lipofectin (Bethesda Research Laboratories). The mixture was overlaid onto a 6-cm plate with COS-7 cells at 70–80% confluence. After incubation at 37 °C for 6 h, 3 ml of Dulbecco's modified Eagle's medium containing 20% FCS was added to each plate, and the plates were incubated at 37 °C for 20 more h. The medium was then replaced with 5 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum. At about 48 h post-transfection, the plate was washed with phosphate-buffered saline twice, and the cells were scraped into 1 ml of phosphate-buffered saline and pelleted. The cell pellet was resuspended in 0.2 ml of phosphate-buffered saline, 0.5% Nonidet P-40 and incubated at 4 °C for 30 min. The cell lysate was then cleared by microcentrifugation at 4 °C for 15 min. One-tenth (20 μl) was used in a standard BLT assay (7). The BLTase activity was expressed as $A_{405}/10^5$ cells/h. Cell extracts from COS-7 cells transfected with the pSRaSD2 vector alone were used to determine the background BLTase level.

RESULTS

Two Different mRNAs Encode Granzyme A—A cDNA library from the CTL line 1E4 was screened with the HF clone (6) to find a full-length cDNA. We isolated and sequenced two different cDNA clones, HF1 and HF2. They are identical at their 5′ ends and throughout the mature granzyme A coding region; however, the HF2 clone has an extra 126 bp inserted 14 bp upstream of the sequence encoding the mature enzyme (Fig. 1*A*). HF1 has an open reading frame that includes a 28-amino acid leader peptide and the active granzyme A sequence. In the HF2 clone, this reading frame is terminated by a stop codon within the insertion. It is followed by another open reading frame that encodes a 25-amino acid peptide and granzyme A. Both of the initiation codons fit well with the

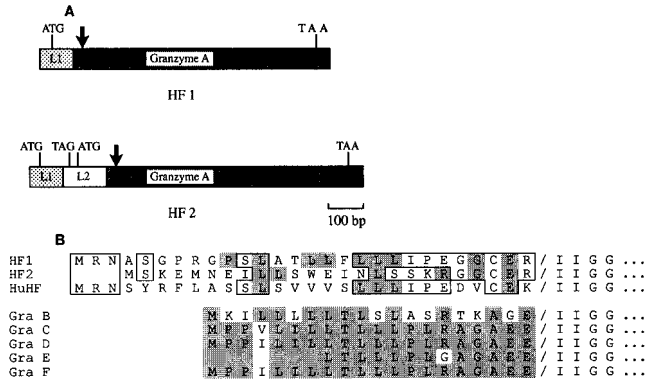

FIG. 1. *Panel A*, schematic representation of the two granzyme A cDNAs. The *arrow* indicates the start of the mature granzyme A coding region. *Panel B*, sequences of the granzyme leader peptides inferred from their cDNA sequences. The *slash* (/) marks the start of the mature active protease. The *boxes* indicate matches of at least 2 of 3 granzyme A residues; the *gray* indicates matches of at least four of all the granzyme sequences. *HuHF*, human granzyme A.

Kozak consensus sequence (35, 36). The consensus sequence is cgccA/GcCAUGgc, where the capital letters are more conserved. The HF1 initiation sequence is AGCCACGATGAG, and the HF2 initiation sequence is ACTTGAGATGAG. The HF1 initiation sequence seems to fit the consensus sequences better than HF2 (see below).

Granzymes B–F all have conserved 20-amino acid leader peptides: an 18-amino acid hydrophobic signal peptide and a 2-amino acid activation peptide (37). The leader peptide of the HF1 form of granzyme A is similar to the leader peptides of the other granzymes. The HF2 leader peptide, however, has less sequence similarity to the other leader peptides (Fig. 1*B*). HF1, like granzymes B–F, has a signal peptide that the von Heijne algorithm (38) predicts will be cleaved to leave a 2-amino acid activation peptide. The predicted cleavage site for the HF2 leader peptide is ambiguous; it could have either a 2- or a 7-amino acid activation peptide. The HF1 leader peptide conforms to the standard structure for signal peptides (20). The putative HF2 signal peptide, in contrast, has its hydrophobic region interrupted by a Glu and an Asn residue. The HF1 sequence is also more related to the leader sequence of human granzyme A (39) than is HF2: the HF1 sequence is 46 and 69% similar to the human sequence at the amino acid and DNA levels, respectively, whereas the HF2 sequence is just 21 and 50% similar (allowing for gaps). These figures suggest that the HF1 form of granzyme A is the mouse homologue of human granzyme A, and it probably localizes to the granules with the other granzymes and perforin. The HF2 form of granzyme A, however, may localize to different compartments in the cells.

To confirm that both the HF1 and HF2 cDNAs represent mRNAs made normally by CTLs, we did a primer extension analysis on RNA from the CTL line AR1, spleen, lymph node, and RAW112 (a B cell lymphoma). No specific granzyme A bands are visible in the RNA from RAW112, spleen, and lymph nodes. The CTL line AR1, however, contains two specific bands (Fig. 2). The strong band (*lower arrow*) is the correct size for an HF1 transcript beginning 29 bp downstream from the TATA box (see below). The faint upper band (*upper arrow*) is 126 bp larger, proving that this CTL line does make a transcript corresponding to the HF2 cDNA. The

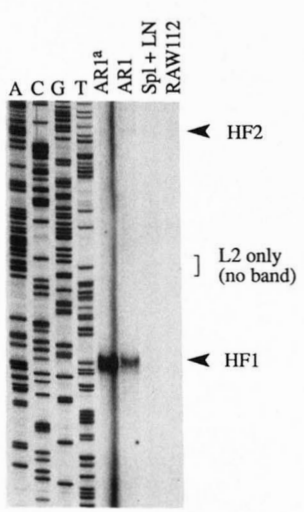

FIG. 2. **Primer extension analysis using a primer (E3) from the third exon.** The *lower arrow* indicates the transcription initiation site for the HF1 mRNA, and the *upper arrow*, the HF2 mRNA. Shown on the *left* are sequencing reactions using the E3 primer on an HF2-containing plasmid. The *bracket* marks the region of the gel where a transcript initiating from the hypothetical second TATA box would appear. *AR1ª*, poly(A) RNA from AR1 cells; *AR1*, total RNA from AR1 cells; *Spl*, spleen; *LN*, lymph node; *RAW112*, a pre-B cell line.

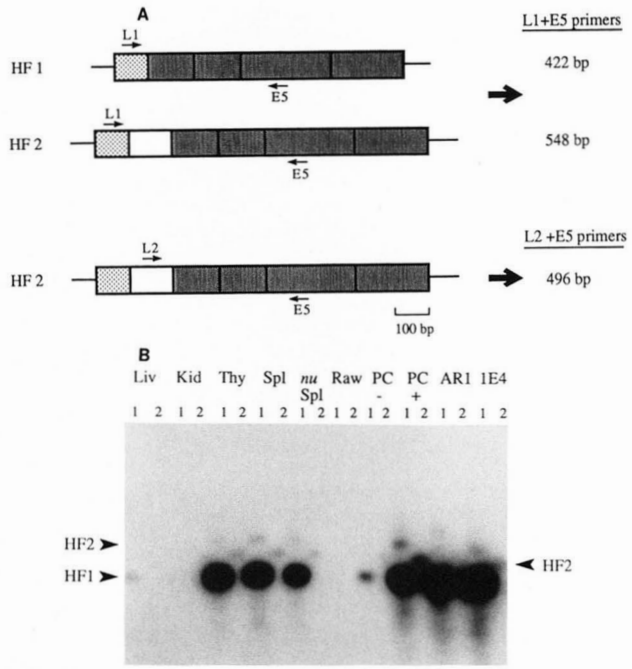

FIG. 3. *Panel A*, schematic representation of the PCR amplification strategy. The *small arrows* indicate the orientation and position of the primers relative to the mRNA. *Panel B*, PCR products from normal tissue and cell line RNAs. Each sample is shown amplified with the L1, E5 primers (*lanes 1*) and the L2, E5 primers (*lanes 2*). *Arrows* indicate the expected sizes for the products. *PC−*, PC60, uninduced; *PC+*, PC60, induced for three days with 25% concanavalin A supernatant; *Liv*, liver; *Kid*, kidney; *Thy*, thymus; *Spl*, spleen; *nu/spl*, nude mouse spleen; *AR1* and *1E4*, two CTL cell lines; *Raw*, RAW112, a pre-B cell line.

relative intensities of these two bands show that the HF1 transcript is the predominant granzyme A message in AR1 cells. Thus, both HF1 and HF2 mRNA are present in the CTL cell line.

Both Granzyme A mRNAs Are Expressed in Normal Lymphoid Tissues—Because the HF2 transcript is at low abundance even in CTL lines, we used the reverse transcriptase/PCR technique (32) to determine the pattern of HF1 and HF2 mRNA distribution in normal lymphoid cells. Single-stranded cDNA synthesized from either total or poly(A)$^+$ RNA was amplified by PCR. We used two pairs of primers (Fig. 3*A*), one of which (L1, E5) amplifies both the HF1 and HF2 forms of the message, giving products of 422 and 548 bp, respectively, and another (L2, E5) that amplifies only the HF2 transcripts, giving a 496-bp product. Fig. 3*B* shows the results from a variety of normal tissues and cell lines. This figure shows that normal lymphocytes from thymus and spleen express both HF1 and HF2. The spleen from a nude mouse, which has natural killer cells but very few T cells, also has both messages.

Activated cytolytic cell lines (PC60, AR1, 1E4) make both HF1 and HF2. Both granzyme A messages are present at a low level in uninduced PC60 cells. Their expression is enhanced concomitantly with increase of BLTase activity and cytotoxicity when this cell line is activated (40).[3]

When amplifying both HF1 and HF2 in a single reaction, using primers that anneal to the first leader (L1) and to a shared downstream sequence (E5), the amplification conditions are identical for both messages. This allows comparison of the relative abundance of the two mRNAs within a sample. Fig. 3*B* shows that the HF1 message is much more abundant than HF2 in all positive samples, but it also shows that all those samples do contain some HF2 message. Therefore, both

[3] L. Su and I. L. Weissman, unpublished results.

HF1 and HF2 are expressed in normal lymphoid tissues, and they are both induced in a cell line with inducible BLTase and cytotoxicity activity.

Both Granzyme A mRNAs Are Expressed in Lymphocytes from Rejecting Skin Allografts—The granzyme A gene is induced in infiltrating lymphocytes when allogeneic heart grafts are being rejected, as shown by *in situ* hybridization (41). Allogeneic skin grafts also evoke a CTL response (42). Therefore, we set up syngeneic and allogeneic skin grafts to test granzyme A mRNA expression in an *in vivo* immune response. The skin grafts were harvested at 8 and 10 days after transplantation, lymphocytes were recovered from the epidermal layer, and mRNA was extracted using a method designed to allow reverse transcriptase/PCR detection of messages from $\leq 10^3$ cells (31). As shown in Fig. 4, the infiltrated lymphocytes from rejecting allografts have a high level of granzyme A messages. This is not because of an increase in the number of cells recovered, as shown by the reverse transcriptase/PCR amplification of actin mRNA from the same cDNA preparations (*lower panel*). Therefore, both forms of granzyme A mRNA are induced during a CTL immune response *in vivo*, although HF1 is present at a much higher level.

Normal C57BL/Ka tail skin has an extremely low level of granzyme A message; a faint band that appears after a long exposure may be caused by the Thy1$^+$ dendritic epidermal cells, which are of thymic origin (43) and can be cytotoxic (44). The negative control, RAW112, never shows any hybridizing band. The syngeneic graft-derived lymphocytes have a slight increase in granzyme A mRNA compared with the ungrafted skin. This may reflect either the activation of the dendritic epidermal cells present in the graft or a T cell response to infection or tissue damage at the graft site.

Both HF1 and HF2 Can Be Translated in Vitro—To test whether both cDNAs can produce the granzyme A protein, we first tested their ability to produce a protein of the expected size in an *in vitro* transcription/translation system. Both cDNAs generate protein products of about 30 kDa (Fig. 5*A*). The HF1 mRNA appears to be more efficient as a template for translation because an equal amount of RNA was used for the translation assay. The Kozak sequences (35, 36) at the translation initiation codon in HF1 or HF2 are different and may account for the difference in translation efficiency.

Expression of the HF1 and HF2 cDNAs was also tested in a transient expression system. The cDNAs were transfected into COS-7 cells under the control of the SV40 early gene promoter. Both cDNAs are transcribed, as shown by reverse transcriptase/PCR analysis of RNA from the transfected cells (data not shown). Whole cell extracts from transfected cells were assayed for BLTase activity, which is very low in untransfected COS-7 cells (data not shown). Cells transfected with the HF1 cDNA produce a high level of BLTase activity (Fig. 5*B*). In contrast, the BLTase activity of cells transfected with HF2 is not significantly above background. This may be because of either inefficient processing of the HF2 granzyme A or inefficient translation of the HF2 message. The leader sequence may also lead to a difference in protein stability. This experiment shows that functional granzyme A can be produced from HF1 but not from HF2 in the transient expression system.

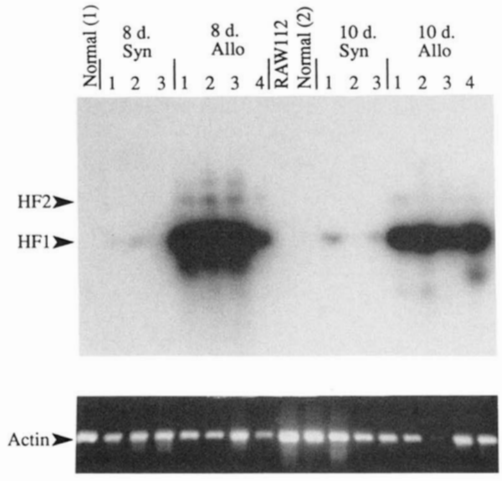

FIG. 4. **PCR products from cells recovered from skin grafts.** *Upper panel*, three syngeneic (*Syn*) grafts and four allogeneic (*Allo*) grafts are shown for each time point. The L1, E5 primer pair was used. *Normal*, normal tail skin from C57BL/Ka mice. *Lower panel*, ethidium bromide-stained agarose gel showing the PCR amplification of actin on the same cDNA preparations.

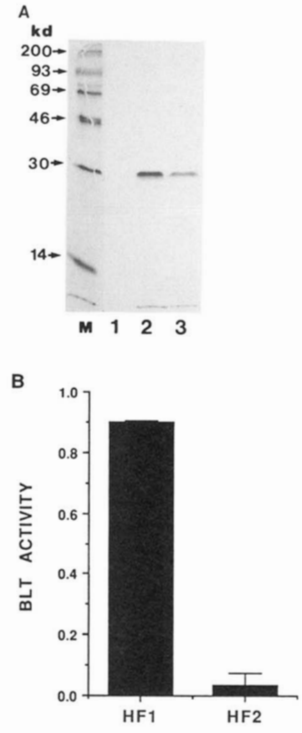

FIG. 5. *Panel A*, *in vitro* translation of HF1 and HF2 mRNA. The transcription, translation, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were carried out as described under "Materials and Methods." The *arrow* indicates the translated products. *Lane 1*, no exogenous RNA control; *lane 2*, translation products from HF1; and *lane 3*; from HF2. *Panel B*, functional expression of HF1 and HF2 in COS-7 cells. BLT assay of transfected cells was carried out as described under "Materials and Methods." Each data point is from an average of triplicates, and each experiment was repeated at least three times. The *error bars* indicate the S.D. *HF1*, cells transfected with pSRaSDHF1; *HF2*, cells transfected with pSRaSDHF2. Cell extracts from cells transfected with the vector (pSRaSD2) were used to determine the background.

The Granzyme A Gene Has Two Separate Leader Exons—To examine the origins of the two mRNAs, we isolated two lEMBL3 mouse genomic clones that cover 18 kb around the granzyme A gene. The gene is composed of six exons spanning 7 kb (Fig. 6*A*). As in many other serine proteases, the residues forming the active site, His-41, Asp-85, and Ser-183, are all on separate exons (45). The genomic organization of granzyme A is most similar to that of granzymes B and C (46), rat mast cell protease II (25), and adipsin (47, 48). This group is a subfamily of the trypsin family of serine proteases, and it is distinguished from trypsin by the position of the third intron (25, 49). However, granzyme A, unlike these other serine proteases, has two different leader exons.

We sequenced all the exons, the exon-intron junctions, and

FIG. 6. *Panel A*, schematic representation of the 18 kb encompassing the granzyme A gene and of the mRNAs made from it. The locations of the active site residues His-41 (*H*), Asp-85 (*D*), and Ser-183 (*S*) are indicated. The restriction map is indicated above in *small letters*. *e EcoRI*; *s*, *SalI*; *b*, *BamHI*; *h*, *HindIII*; *x*, *XhoI*. *Panel B*, DNA sequences of the granzyme A gene that includes 500 bp upstream of the gene, all of the exons, and some of the intron sequences. The exon-intron boundaries are denoted by a *slash*. The TATA box is *underlined*, and the transcription initiation site is marked with a *capital letter*. The inferred amino acid sequences are indicated below their corresponding DNA sequences, and an *underlined* TAG marks the stop codon that halts transcription from the *L1* methionine in the HF2 message. A TATA-like sequence in the first intron is indicated by *italic letters*. Initrons 3, 4, 5 are not completely sequenced. The missing length of sequences is indicated in *brackets*. The *star* marks the stop codon (TAA), and the polyadenylation signal (AATAAA) is *underlined*.

the region 5′ of the granzyme A gene. Fig. 6*B* gives the sequences 5′ of the transcription initiation site and the first two exons, the first intron, the other exons and part of the other introns. The first exon encodes 23 amino acids of the HF1 leader peptide. The second exon also encodes a partial leader peptide, and it is alternatively spliced: if it is excluded the HF1 message is produced, and if it is included the HF2 message is made. Most of the granzyme A splice donor and acceptor sites conform to the consensus sequence (Fig. 6*B*). The splice acceptor site of the second leader exon differs from the consensus sequence, however, in that a stretch of 5 Ala residues separates the polypyrimidine tract from the rest of the splice acceptor. Purine-rich sequences at the 3′ splice acceptor are present naturally in the introns preceding alternatively spliced exons in genes from *Drosophila* to human (50). Thus, this divergence from the consensus splice sequence may be the means for regulating the production of the two forms of granzyme A.

The most common mechanism for making two different amino termini on a protein is the use of different transcription initiation sites for the mRNAs (35, 51). A TATA promoter element (52) lies 29 bp upstream of the transcription initiation site identified by primer extension analysis. In addition, there is a TATA-like sequence in the first intron (*italicized* in Fig. 6*B*) that could be the promoter for an mRNA beginning with the second exon. However, the primer extension analysis in Fig. 2 shows no transcript beginning 26–34 bp downstream of this site (*bracket*). The lack of detectable messages that begin with the second exon implies that alternative splicing is the only mechanism that creates an mRNA encoding the second leader peptide for granzyme A.

Examining the 500 bp of genomic sequences 5′ of granzyme A (Fig. 6*B*) reveals no significant match to the consensus CAAT sequence, the GC box sequences, nor to the other consensus promoter/enhancer sequences compiled by Wingender (53). Comparison with putative promoters of the other granzyme genes (54, 55) and the perforin gene (56) shows some limited matches but no significantly homologous regions. The putative regulatory sequences conserved between human and mouse perforin genes (56) are not conserved in the 500-bp promoter region of the mouse granzyme A gene. The granzyme A gene, whose expression is inducible and cell type-specific (6, 57), may provide a good system to study gene regulation.

DISCUSSION

In an attempt to clone a full-length granzyme A cDNA, two clones were isolated and characterized. They share a common 5′ end and the mature protease encoding region, but the second cDNA (HF2) has an insertion of 126 bp in the region encoding the putative leader peptide. Analyses of CTL *in vitro* and immune infiltrates *in vivo* confirm the presence of both messages. They are both induced during activation of T cells (presumably CTL) *in vitro* and *in vivo*, and granzyme A can be produced from both cDNAs in an *in vitro* transcription/translation system. Therefore, the HF2 cDNA appears to be a *bona fide* message expressed in cytolytic cells.

The genomic structure of the mouse granzyme A gene is similar to other trypsin family genes; however, it is the only one in the family that has two leader exons. Two granzyme A mRNAs, HF1 and HF2, are generated by alternative splicing of the second exon. Analysis of the splicing junctions reveals that the second exon has a polypurine stretch adjacent to the splice acceptor site. In a study on splicing of the SV40 large T and small t antigens, the insertion of a polypurine tract between the polypyrimidine tract and the splice acceptor had no effect on the splicing efficiency, but it has a considerable effect on the pattern of alternative splicing (50). Therefore, the generation of HF2 may be regulated by some *trans*-acting factor binding near this splice acceptor site. The granzyme A gene may provide a good system to study alternative splicing,

as the message is very abundant and one message is predominantly spliced over the other.

The putative promoter region of the granzyme A gene has a typical TATA box 29 bp upstream of the CAP site (Fig. 6*B*). Comparison with promoter regions of genes encoding the CTL granular proteins perforin (56) and granzyme B (54) reveals no significant conserved sequence motifs. More detailed studies of more upstream sequences are in progress to characterize the regulatory elements important for its induction in activated cytolytic cells.

The cellular location and expression pattern of granzyme A imply that it may be involved in the killing process. Studies with protease inhibitors also indicate involvement of proteases during cell-mediated killing (13–15). Recent studies with purified perforin and granzyme A have shown that granzyme A may be important in inducing target cell nuclear and DNA degradation, an event associated with CTL-mediated cytolysis (18). Specific inhibition of the granzyme A function in CTL cells either by an HF antisense RNA[2] or by a dominant negative mutant of the granzyme A gene[3] indicated that granzyme A is required for efficient killing of target cells.

The granule-mediated cell lysis model has been challenged by the following observations. First, some *in vivo* primed CTL do not contain any cytolytic granules but can kill target cells quite efficiently (59). Second, some CTL can kill target cells in the absence of extracellular calcium (60–62), which is required for granule exocytosis. Therefore, mechanisms other than lytic granule exocytosis have been proposed (17, 63). Whether these represent further redundance pathways to cytolysis to ensure lysis of target cells or they also use the granule exocytosis pathway (therefore requiring some other function for granule exocytosis events) is not yet clear. The demonstration that granzyme A can be encoded by two mRNAs with different leader sequences implies that the same type of effector molecules may be employed in different cellular compartments and/or different cytolytic pathways. It is conceivable that the HF2 granzyme A may be stored in an inactive form in the nongranulated CTL cells and is activated during the killing processes. The HF2 message may be too rare to be detected by Northern blot analysis in those cells (59). The reverse transcriptase/PCR assay used in this study might be employed to address this issue. It is also conceivable that HF2 message expression leads to a different function in the life history of cytolytic cells. For example, *in vitro* (18) study strongly supports the notion that granzyme A can stimulate target cell DNA fragmentation, presumably getting into such target cells by perforin pores. If HF2 mRNA is expressed late in the life history of killer cells and results in release of granzyme A into the cytosol, apoptosis-associated nuclear fragmentation might result. In that view, the disappearance of cytolytic cells in the course of an immune response may be triggered by a switch in the splicing mode of HF RNA. Of course this speculation is only one of several possible explanations for the presence of HF1 and HF2 mRNA forms but is valuable in that it is a testable hypothesis. It will also be of interest to study if other CTL granular proteins are also encoded by different mRNAs. Interestingly, a different cDNA encoding human granzyme B with a different leader peptide has been reported recently (58).

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