FEBS Letters 364 (1995) 268–271 FEBS 15479

# Cloning of cDNA for human granzyme 3

Melinda M. Przetak, Sienna Yoast, Brian F. Schmidt*

*Khepri Pharmaceuticals Inc., 260 Littlefield Avenue, South San Francisco, CA 94080, USA*

Received 6 March 1995

**Abstract A serine protease gene was cloned from cDNA prepared from tissue isolated from human ascites. The gene codes for a protein of 264 amino acids, identified as human granzyme 3 by the N-terminal amino acid sequence. Granzyme 3 has the expected features of the chymotrypsin family of serine proteases, and is closely related to other human granzymes (40–45% identity). However, the closest granzyme 3 homologue is the recently characterized rat tryptase, RNK-Tryp-2 (75% identity). From Northern blots, granzyme 3 appears to be highly expressed in peripheral blood leukocytes, spleen, thymus, and lung tissues.**

*Key words:* Serine protease; Tryptase; Granzyme; Cytoplasmic granule

## 1. Introduction

A group of related serine proteases have been isolated from the cytoplasmic granules of cytotoxic lymphocytes and natural killer cells [1,2]. Although, these granzymes appear to have a role in the cytolytic event, their precise function has yet to be elucidated [3]. Evidence suggests that they may be involved in the degradation of extracellular matrix, inhibition of viral and/or bacterial infections, or have a specific, but as yet undefined, target that triggers apoptosis [4–6]. Currently, the sequence for four human granzyme genes have been reported [7–13], and the substrate specificity for three of them have been characterized [13–15]. These granzymes belong to the chymotrypsin family of serine proteases [16], and form a subfamily with other human proteases isolated from cytoplasmic granules (cathepsin G and mast cell chymase). A fifth human granzyme, granzyme 3, has been purified and characterized as having trypsin-like specificity [17,18], but the cloning of this gene has not been reported.

## 2. Materials and methods

All custom synthesized oligonucleotides were purchased from Operon Technologies. Tissue isolated from human ascites was generously provided by Bernard Babior (Scripps Research Institute). A cDNA library from this tissue, constructed in the Uni-ZAP XR vector, was obtained from Stratagene Cloning Systems.

The supernatant of the amplified $\lambda$ phage cDNA library ($1.3\times10^{10}$ pfu/ml) was screened for serine proteases (chymotrypsin family) using PCR with primers complementary to active site histidine and serine sequences. Specifically, 1 $\mu$l of phage supernatant was PCR amplified with two degenerate DNA primer pools (2 $\mu$M of each pool, GTNTTRTCNGCNGCNCAYTGY and AKNGGNCCNCCNGARTCNC) using 1 $\mu$l (5 U/$\mu$l) of Taq Polymerase (Perkin/Elmer). The final volume was 50 $\mu$l in PCR Buffer (Perkin/Elmer) with a 40 $\mu$l mineral oil overlay. The thermal cycling followed a 'touchdown' protocol [19] with initial incubations of 97°C for 5 min and then 92°C for 3 min prior to the addition of the Taq polymerase. Further denaturation steps were performed at 94°C for 30 s and extensions were done at 72°C for 30 s except for the final extension which was for 10 min at 72°C. Annealing was performed for 30 s; in the first 5 cycles at 65°C, then the next 5 cycles at 60°C, 55°C for the next 5 cycles, and finally at 50°C for the final 35 cycles. The PCR reaction products were fractionated on a 5% polyacrylamide gel and a band corresponding to 470 base pairs was cut out of the gel and extracted by electroelution. The purified fragment (phenol/chloroform extracted, ethanol precipitated) was cloned into the vector pCRII using a TA cloning kit (Invitrogen). Individual white colonies were picked from Luria-Burtani agar plates containing 40 $\mu$g/ml X-gal and 50 $\mu$g/ml carbenicillin for plasmid selection. After growth in liquid medium, plasmids were isolated (Wizard Minipreps, Promega) and screened for the correct insert on 5% polyacrylamide gels after digestion with *Eco*RI. Plasmids with the correct size inserts were ethanol precipitated (to improve template quality), dissolved in 50 $\mu$l of water, and the inserts sequenced using M13-forward and M13-reverse primers (Promega). The opposite strands were sequenced using primers complementary to sequences within the protease gene (CCTCAATCAAATGATATCATGCTGG and CAGTGACTTCTCGCAGGGTGTCAG). DNA was sequenced using an ABI 373A DNA Sequencer and a DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems).

The remaining 5′ portion of the granzyme 3 gene was PCR amplified using the primers M13-reverse, T3 (Promega), or M13–85r (CTTTATGCTTCCGGCTCGTATGTTGTGTGG) that hybridize to the cDNA vector (pBluescript in Uni-Zap XR, Stratagene) versus two primers that hybridize inside the gene (AACCACAGTGGGAGACTGGCCT or GGAGGCCTCATTCTTTGAGAGAGAGTGTGC). In a similar way, the 3′ portion of the gene was obtained by PCR amplifications using vector primers T7 (Promega) or M13-forward versus a gene primer (GGAGATGCCAAAGGCCAGAAGGAT). DNA fragments corresponding to the 3′ and 5′ portion of the gene were cloned and sequenced as above. Finally, the entire mature portion of the gene was PCR amplified with primers (GTGTGTTATGCATATGCCATTATTGGAGGGAAAGAAGTGTCACCTCAT and CCAATAAGGATCCGAATTCATTAATTTGTATGAGGCGGGACAAGGTTG), cloned in pCRII, and sequenced. Due to the error frequency of Taq polymerase, at least six different clones from each portion of the gene were sequenced to determine the final consensus sequence.

Human tissue Northern blots (Clontech) were probed with a *Nsi*I-*Bam*HI fragment isolated from pCRII corresponding to the gene region that codes for the mature granzyme 3. Prior to hybridization, this fragment was labeled with $^{32}$P using a random priming kit (Amersham).

Computer alignments of protein and DNA sequences were made using the Pileup and Gap programs (Genetics Computer Group). Unless stated otherwise, all numbering of amino acids residues are based on the bovine chymotrypsin sequence [20].

## 3. Results and discussion

A serine protease gene, belonging to the chymotrypsin family [16], was discovered by using PCR to screen a cDNA library of human tissue isolated from ascites. A putative start codon was identified near the 5′ end of the full-length cDNA consensus sequence, and the following open reading frame codes for a serine protease of 264 amino acids (Fig. 1). A polyadenylation

*Corresponding author. Fax: (1) (415) 794-3599.
E-mail: schmidt@khepri.com

*Abbreviations:* PCR, polymerase chain reaction; X-gal, 5-bromo-4-chloro-3-indolyl-$\beta$-D-galactopyranoside.


*SSDI* 0014-5793(95)00407-6

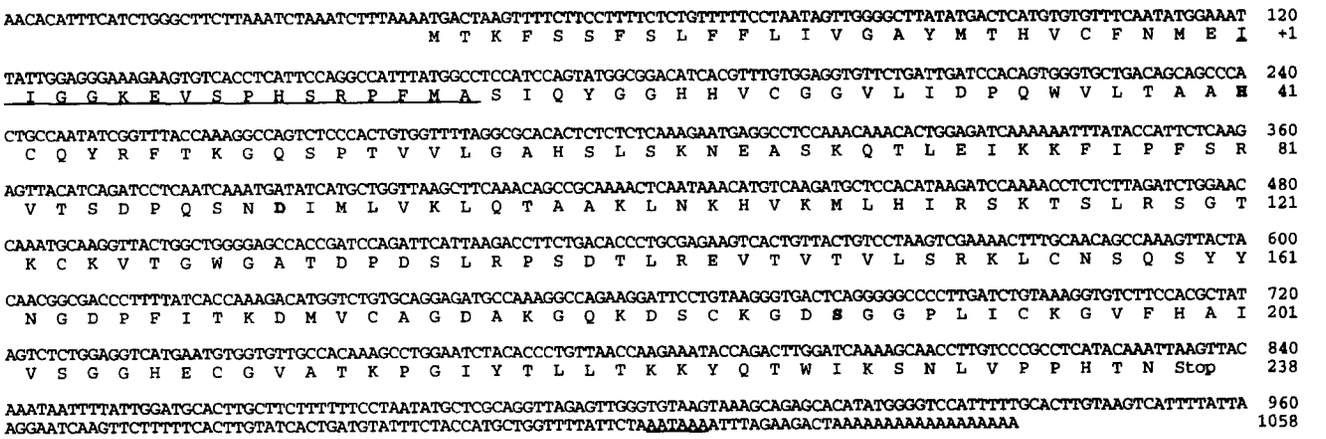


Fig. 1. The nucleotide sequence of the granzyme 3 gene cDNA and the translated protein sequence. In the nucleotide sequence the polyadenylation signal site is underlined. In the amino acid sequence, the N-terminal sequence of the mature protease determined by protein sequencing [17] is underlined and the catalytic triad residues are in bold.

signal and a polyA tail were found at the 3′ end of the cDNA sequence (Fig. 1). This gene was identified as granzyme 3 since the predicted N-terminal sequence of the mature protein is identical to that reported for human granzyme 3 (Fig. 1) [17]. Preceding the mature protease sequence is a typical signal sequence [21] most likely terminated by a short propeptide that ends in an acidic residue (E at −1). This propeptide sequence is common to many serine proteases stored in cytoplasmic granules (Fig. 2) [1,2] and probably reflects activation by a common processing peptidase.

Human granzyme 3 has about 40–45% identity to other members of the human granzyme group (Table 1), and has somewhat lower identity to bovine chymotrypsin (37%). However, its closest homologue (75% identity) is the recently cloned rat tryptase, RNK-Tryp-2 [22]. Thus, these two tryptases may play similar roles in their respective species. A human equivalent of RNK-Tryp-2 has reportedly been cloned, but it has not been described as being granzyme 3 and no sequence data was provided [23].

In the granzyme 3 amino acid sequence, the predicted catalytic triad (H57, D102, and S195) and adjacent sequences fit the consensus model for the chymotrypsin family of serine proteases [16]. The N-terminal sequence is also nearly identical to that characteristic of the granzyme group of serine proteases (IIGGXXXXPHSRPYMA, Fig. 2) [1]. This sequence defines the arm whose N-terminal amino acid (I16) forms an ion pair with D194, after propeptide cleavage, generating the active form of the protease [24]. In addition, the fourth member of the catalytic group, S214 [25], and other residues predicted to be involved in defining the configuration of the active site (G43, G44, G140, W141, G142, L155) [24], are also conserved in granzyme 3.

There is an aspartate residue in the bottom of the putative S1 binding site (D189) and glycines on both sides of the pocket (G216 and G226) as found in other proteases with trypsin-like specificity. This agrees with the fact that purified granzyme 3 cleaves a substrate with lysine at P1 [17,18]. It is unusual that granzyme 3, like RNK-Tryp-2 [22], has a glycine at residue 215, the top of the S1 site (usually a tryptophan, phenylalanine, or tyrosine, Fig. 2), Thus, granzyme 3 may have a unique substrate specificity compared to other tryptases, distinguishing its function from that of the other human granzyme tryptase, granzyme A.

It can also be predicted that granzyme 3 has four disulfide bonds typically conserved in many members of the chymotrypsin family (42–58, 136–201, 168–182, and 191–220, Fig. 2). However, the C-terminal disulfide (191–220) is missing in most other proteases of the granzyme group (Fig. 2) [1]. Since all available cysteines would be predicted to form disulfide bonds, granzyme 3 would not have any remaining free cysteines which

Table 1
Percent identity among selected serine proteases

| Protease[a] | % Identity of protease pair | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | hGran3 | rTryp2 | hGranA | hChym | hMetas | hGranB | hGranH | hCatG |
| rTryp2 | 75 | | | | | | | |
| hGranA | 45 | 49 | | | | | | |
| hChym | 41 | 40 | 43 | | | | | |
| hMetas | 40 | 42 | 40 | 36 | | | | |
| hGranB | 42 | 41 | 41 | 52 | 41 | | | |
| hGranH | 41 | 42 | 44 | 49 | 42 | 71 | | |
| hCatG | 39 | 40 | 41 | 52 | 42 | 57 | 56 | |
| bChymA | 37 | 39 | 33 | 36 | 33 | 32 | 31 | 33 |

[a] Protease abbreviations same as in Fig. 2.

could form intermolecular disulfide linkages as in the formation of granzyme A dimers [4]. Although most granzymes are highly glycosylated [1], there are no consensus sequences indicative of N-linked glycosylation sites in granzyme 3.

In addition to granzyme 3, we have cloned a number of serine proteases associated with cytoplasmic granules (granzyme B, granzyme H, Met-ase, and mast cell chymase) from the cDNA made from ascitic tissue (data not shown). Thus, as one might expect, human ascites is a good source of cells involved in the immune response. In Northern blots, the granzyme 3 gene hybridizes strongly to a band of the expected size (1–1.1 kb) in mRNA isolated from adult human lung, spleen, thymus, and peripheral blood leukocytes (Fig. 3). This is not surprising since these tissues should be good sources of cytolytic lymphocytes. Weaker hybridization signals were observed in mRNA from heart, liver, prostate, ovary, small intestine, and colon. Due to the potential for cross-hybridization to other serine protease genes, which appears to occur in mRNA isolated from the pancreas (Fig. 3), one cannot unambiguously assign the hybridization band seen in Northern blots to the granzyme 3 mRNA.

Due to the low levels of granzyme 3 found in activated killer cells [17,18], recombinant expression of the cloned granzyme 3 gene may be the best way to produce enough protein for enzymatic characterization.

*Acknowledgments:* We thank L.M. Babé, D.A. Estell, M.E. McGrath, and D.G. Payan for helpful discussions and advice.

```
          -26                              1
hGranB              MQPILLLLAFLLLPRADAGEIIGGHEAKPHSRPYMAYLMIW
hGranH              MQPFLLLLAFLLTPGAGTEEIIGGHEAKPHSRPYMAFVQFL
hCatG               MQPLLLLLAFLLPTGAEAGEIIGGRESRPHSRPYMAYLQIQ
hChym             MLLLPLPLLLFLLCSRAEAGEIIGGTECKPHSRPYMAYLEIV
hGran3      MTKFSSFSLFFLIVGAYMTHVCFNMEIIGGKEVSPHSRPFMASIQY.
rTryp2       MSFSSSALVFLVAGIYMSSESFHTEIIGGREVQPHSRPFMASIQY.
hGranA  MRNSYRFLASSLS.VVVSLLLIPEDVCEKIIGGNEVTPHSRPYMVLLSL.
hMetas      MEACVSSLLVLALGALSVGSSFGTQIIGGREVIPHSRPYMASLQ..
bChymA                  CGVPAIQPVLSGLSRIVNGEEAVPGSWPWQVSLQ..
                        1

                                      *        50
hGranB  DQKSLKR.CGGFLIQDDFVLTAAHC.....WGSSINVTLGAHNIKEQEPT
hGranH  QEKSRKR.CGGILVRKDFVLTAAHC.....QGSSINVTLGAHNIKEQERT
hCatG   SPAGQSR.CGGFLVREDFVLTAAHC.....WGSNINVTLGAHNIQRRENT
hChym   TSNGPSKFCGGFLIRRNFVLTAAHC.....AGRSITVTLGAHNITEEEDT
hGran3  ...GGHHVCGGVLIDPQWVLTAAHCQYRFTKGQSPTVVLGAHSLSKNEAS
rTryp2  ...RGKHICGGVLIHPQWVLTAAHC...YSRGHSPTVVLGAHSLSKNEPM
hGranA  ...DRKTICAGALIAKDWVLTAAHCNL....NKRSQVILGAHSITREEPT
hMetas  ..RNGSHLCGGVLVHPKWVLTAAHCLAQ..RMAQLRLVLGLHTLDSPGLT
bChymA  .DKTGFHFCGGSLINENWVVTAAHCGV....TTSDVVVAGEFDQGSSSEK
                        50

                                      *        100
hGranB  QQFIPVKRPIPHPAYNP.KNFSNDIMLLQLERKAKRTRAVQPLRLPSNKA
hGranH  QQFIPVKRPIPHPAYNP.KNFSNDIMLLQLERKAKWTTAVRPLRLPSSKA
hCatG   QQHITARRAIRHPQYNQ.RTIQNDIMLLQLSRRVRRNRNVNPVALPRAQE
hChym   WQKLEVIKQFRHPKYNT.STLHHDIMLLKLKEKASLTLAVGTLPFPSQFN
hGran3  KQTLEIKKFIPFSRVTS.DPQSNDIMLVKLQTAAKLNKHVKMLHIRSK.T
rTryp2  KQTFEIKEFIPFSGFKS.G..TNDIMLIKLRTAAELNKHVQLLHLRSK.N
hGranA  KQIMLVKKEFPYPCYDP.ATREGDLKLLQLTEKAKINKYVTILHLPKKGD
hMetas  ...FHIKAAIQHPRYKPVPALENDLALLQLDGKVKPSRTIRPLALPSKHQ
bChymA  IQKLKIAKVFKNSKYNSL.TINNDITLLKLSTAASFSQTVSAVCLPSASD
                        100

                                              150
hGranB  QVKPGQTCSVAGWGQTAPLG.KHSHTLQEVKMTVQEDRKCESDLRHYYDS
hGranH  QVKPGQLCSVAGWG.YVSMS.TLATTLQEVLLTVQKDCQCERLFHGNYSR
hCatG   GLRPGTLCTVAGWGR.VSMR.RGTDTLREVQLRVQRDRQCLRIF.GSYDP
hChym   FVPPGRMCRVAGWGRTGVLK.PGSDTLQEVKLRLMDPQACSHF..RDFDH
hGran3  SLRSGTKCKVTGWGATDPDSLRPSDTLREVTVTVLSRKLCNSQSYYNGDP
rTryp2  YIRDGTKCQVTGWGSTKPDVLTTSDTLQEVTVTIISRKRCNSQSYYNHKP
hGranA  DVKPGTMCQVAGWGRTH.NSASWSDTLREVNITIIDRKVCNDRNHYNFNP
hMetas  VVAAGTRCSMAGWGLTH.QGGGLSRVLRELDLQVLDTRMCNNSRF..WNG
bChymA  DFAAGTTCVTTGWGLTRYTNANTPDRLQQASLPLLSNTNCKK....YWGT
                        150

                                      *               200
hGranB  TIE...LCVGDPEIKKTSFKGDSGGPLVCN..K..CAQGIVSYGR....N
hGranH  ATE...ICVGDPKKTQTGFKGDSGGPLVCK..D..VAQGILSYGN....K
hCatG   RRQ...ICVGDRRERKAAFKGDSGGPLLCN..N..VAHGIVSYGK....S
hChym   NLQ...LCVGNPRKTKSAFKGDSGGPLLCA..G..VAQGIVSYGR....S
hGran3  FITKDMVCAGDAKGQKDSCKGDSGGPLICK..G..VFHAIVSGG..HECG
rTryp2  VITKDMICAGDRRGEKDSCKGDSGGPLICK..G..VFHALVSGG..YKCG
hGranA  VIGMNMVCAGSLRGGRDSCNGDSGSPLLCE..G..VFRGVTSFGLENKCG
hMetas  SLSPSMVCLAADSLDQAPCKGDSGGPLVCG..KGRVLAGVLSFSS.RVCT
bChymA  KIKDAMICAGA..SGVSSCMGDSGGPLVCKKNGAWTLVGIVSWGS..STC
                                    200

                                     238
hGranB  NGMPPRACTKVS.SFVHWIKKTMKRY
hGranH  KGTPPGVYIKVS.HFLPWIKRTMKRL
hCatG   SGVPPEVFTRVS.SFLPWIRTTMRSFKLLDQMETPL
hChym   DAKPPAVFTRIS.HYRPWINQILQAN
hGran3  VATKPGIYTLLTKKYQTWIKSNLVPPHTN
rTryp2  ISNKPGVYTLLTKKYQTWIKSKLAPSSAH
hGranA  DPRGPGVYILLSKKHLNWIIMTIKGAV
hMetas  DIFKPPVATAVA.PYVSWIRKVTGRSA
bChymA  STSTPGVYARVT.ALVNWVQQTLAAN
                                   245
```

Fig. 2. An alignment of human serine proteases of the granzyme subfamily; granzyme B (hGranB) [7,8], granzyme H (hGranH) [10–12], cathepsin G (hCatG) [26], mast cell chymase (hChym) [27], granzyme 3 (hGran3), granzyme A (hGranA) [7], and Met-ase (hMetas) [13]. The rat granule tryptase, RNK-Tryp-2 (rTryp2) [22], and bovine chymotrypsin A (bChymA) [20] were added for comparative purposes. The numbering on the top and bottom of the alignment correspond to granzyme 3 and bovine chymotrypsin, respectively, and the catalytic triad residues are marked by asterisks.

## References

[1] Haddad, P., Jenne, D.E., Krähenbühl, O. and Tschopp, J. (1993) in: Cytotoxic Cells. Recognition, Effector Function, Generation, and Methods (Sikovsky, M and Henkart, P. eds.) pp. 251–262, Birkhäuser, Boston.
[2] Simon, M.M., Ebnet, K. and Kramer, M.D. (1993) in: Cytotoxic Cells Recognition, Effector Function, Generation, and Methods (Sikovsky, M and Henkart, P., eds.) pp. 278–294, Birkhäuser, Boston.
[3] Berke, G. (1994) Ann. Rev. Immunol. 12, 735–773.
[4] Simon, M.M. and Kramer, M.D. (1994) Methods Enzymol. 244, 68–79.
[5] Peitsch, M.C. and Tschopp, J. (1994) Methods Enzymol. 244, 80–87.
[6] Smyth, M.J., Browne, K.A., Thia, K.Y.T., Apostolidis, V.A., Kershaw, M.H. and Trapani, J.A. (1994) Clin. Exp. Pharm. Phys. 21, 67–70.
[7] Gershenfeld, H.K. and Weissman, I.L. (1986) Science 232, 854–858.
[8] Schmid, J. and Weissman, C. (1987) J. Immunol. 139, 250–256.
[9] Tranpani, J.A., Klein, J.L., White, P.C. and Dupont, B. (1988) Proc. Natl. Acad. Sci. USA 85, 6924–6928.
[10] Meier, M., Kwong, P.C., Frégeau, C.J., Atkinson, E.A., Burrington, M., Ehrman, N., Sorensen, O., Lin, C.C., Wilkins, J. and Bleackley, R.C. (1990) Biochemistry 29, 4042–4049.
[11] Klein, J.L., Selvakumar, A., Trapani, J.A. and Dupont, B. (1990) Tissue Antigens 35, 220–228.
[12] Haddad, P., Jenne, D., Tschopp, J., Clément, M.-V., Mathieu-Mahul, D. and Sasportes, M. (1991) Int. Immunol. 3, 57–66.
[13] Smyth, M.J., Sayers, T.J., Wiltrout, T., Powers, J.C., and Trapani, J.A. (1993) J. Immunol. 151, 6195–6205.
[14] Odake, S., Kam, C.-M., Narasimhan, L., Poe, M., Blake, J.T., Krahenbuhl, O., Tschopp, J. and Powers, J.C. (1991) Biochemistry 30, 2217–2227.
[15] Poe, M., Blake, J.T., Boulton, D.A., Gammon, M., Sigal, N.H., Wu, J.K. and Zweerink, H.J. (1991) J. Biol. Chem. 266, 98–103.
[16] Rawlings, N.D. and Barrett, A.J. (1994) Methods Enzymol. 244, 19–61.
[17] Hameed, A., Lowrey, D.M., Lichenheld, M. and Podack, E.R. (1988) J. Immunol. 141, 3142–3147.
[18] Hanna, W.L., Zhang, X., Turbov, J., Winkler, U., Hudig, D. and Froelich, J. (1993) Protein Expression Purif. 4, 398–404.
[19] Don, R.H., Cox, P.T., Wainwright, B.J., Baker, K. and Mattick, J.S. (1991) Nucleic Acids Res. 19, 4008.
[20] Birktoft, J.J., Blow, D.M., Henderson, R. and Steitz, T.A. (1969) Phil. Trans. R. Soc. Lond. B 257, 67–76.

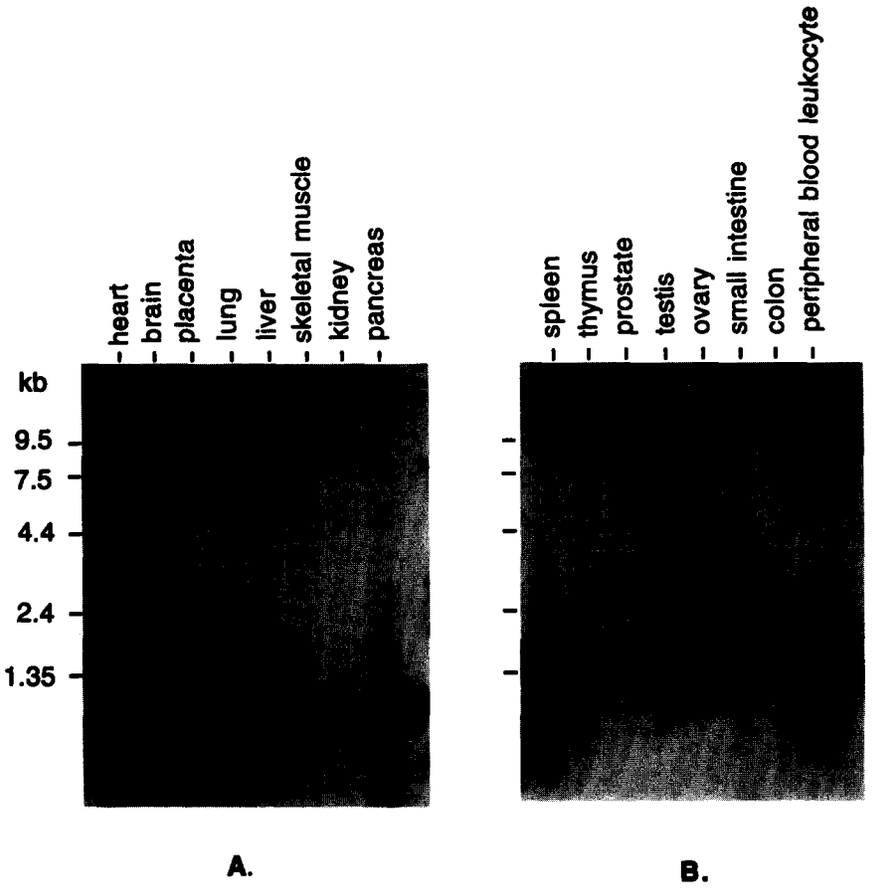


Fig. 3. Northern blots of mRNA from the adult human tissues shown probed with the granzyme 3 gene.

[21] von Heijne, G. (1992) in: Genetic Engineering (Setlow, J.K., ed.) vol. 14, pp. 1–11, Plenum Press, New York.
[22] Sayers, T.J., Wiltrout, T.A., Smyth, M.J., Ottaway, K.S. Pilaro, A.M., Sowder, R., Henderson, L.E., Sprenger, H. and Lloyd, A.R. (1994) J. Immunol. 152, 2289–2296.
[23] Baker, E., Sayers, T.J., Sutherland, G.R. and Smyth, M.J. (1994) Immunogenetics 40, 235–237.
[24] Rypniewski, W.R., Perrakis, A., Vorgias, C.E. and Wilson, K.S. (1994) Protein Eng. 7, 57–64.
[25] McGrath, M.E., Vásquez, J.R., Craik, C.S., Yang, A.S., Honig, B. and Fletterick, R.J. (1992) Biochemistry 31, 3059–3064
[26] Salvesen, G., Farley, D., Shuman J., Przybyla, A., Reilly, C. and Travis, J. (1987) Biochemistry 26, 2289–2293.
[27] Jenne, D.E. and Tschopp, J. (1991) Biochem J. 276, 567–568.