

Structure and Expression of Neutrophil Gelatinase cDNA

IDENTITY WITH TYPE IV COLLAGENASE FROM HT1080 CELLS*

(Received for publication, February 28, 1992)

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Neutrophils synthesize and store intracellularly a 92-kDa type IV collagenase (gelatinase), the primary structure of which is unknown. We designed a primer based on the highly conserved cysteine-switch region of metalloproteinases and employed the polymerase chain reaction to generate a probe of the human neutrophil gelatinase (HNG) gene. This probe was used to clone the cDNA encoding HNG by screening a chronic granulocytic leukemia cDNA library. *In vitro* translation of the cDNA-derived HNG mRNA yielded a major product of 78 kDa and smaller autolytically activated or degraded products, all of which were recognized by anti-HNG antibody. The HNG cDNA sequence is nearly identical to that encoding a 92-kDa gelatinase secreted by HT1080 cells. In addition, primer extension and S1 analysis reveal that the above two gelatinase transcripts have similar initiation sites. The HNG cDNA hybridized to a 2.8-kilobase mRNA from chronic granulocytic leukemia cells. HNG mRNA expression was absent from uninduced HL60 cells and from HL60 cells induced to granulocytic maturation with Me₂SO. However, unlike other neutrophil secondary granule genes, HNG mRNA was detected in HL60 cells induced to monocytic maturation with 12-*O*-tetradecanoylphorbol 13-acetate. This suggests that the HNG gene may be subject to differential control pathways in two related but distinct hematopoietic lineages.

The degradation and remodeling of the extracellular matrix associated with human growth, healing, tumor invasion, and a variety of pathologic disorders is catalyzed by the matrix metalloproteinases (MMP)¹ (1), a family of homologous proteinases encoded for by related genes (2–8). These enzymes are collectively capable of degrading all of the structural

macromolecules that comprise the matrix, although they differ in their individual specificities toward protein substrates. The MMPs also differ in their molecular weights and domain structures (9) but share homologous cysteine-switch and zinc-binding regions (2–8, 10). They are secreted as zymogens which undergo extracellular activation (10) and are inhibited by tissue inhibitors of metalloproteinases (11–13). Important members of the human MMP family include the fibroblast-type (14) and the neutrophil-type (6, 7) interstitial collagenases, three distinct stromelysins (3–5, 26), the 72- and 92-kDa gelatinases (2, 8), and PUMP-1 (26).

Widespread interest in the gelatinases, originally named for their potent hydrolytic action on gelatins, stems from their ability to degrade the type IV collagen found in basement membranes. Since these membranes are a barrier for migrating cells, several investigators have suggested that gelatinases can facilitate tumor invasion, metastasis, and angiogenesis (9, 15). The 72-kDa gelatinase is secreted by proliferating skin fibroblasts, H-ras-transformed bronchial epithelial cells, and SV40-transformed lung fibroblasts (2). The 92-kDa enzyme secreted by SV40-transformed lung fibroblasts resembles the 72-kDa type in primary structure and substrate specificity but has an additional 54-residue proline-rich domain (8). Normal macrophages, induced U937 monocytic leukemia cells, induced HT1080 fibrosarcoma cells, and cultured human keratinocytes secrete an identical 92-kDa gelatinase (8). However, it is not known whether the 92-kDa gelatinase produced and stored intracellularly by neutrophils (16) is identical to that secreted by these other cells. The interstitial collagenases produced by fibroblasts (14) and neutrophils (6, 7) are, in fact, distinct enzymes.

In this study, the full-length cDNA encoding human neutrophil gelatinase (HNG) has been isolated from a chronic granulocytic leukemia (CGL) cDNA library. Comparisons with the HT1080 gelatinase cDNA were made by nucleotide sequencing, primer extension, and S1 analysis. The expression of HNG in the context of its identity as a neutrophil secondary granule protein was also examined. Secondary granule protein gene expression is thought to be coordinately regulated at the level of mRNA transcription and is restricted to those stages of neutrophil maturation associated with secondary granule formation, namely the myelocyte stage and beyond (17). HL60 cells are a human leukemia cell line arrested at the myeloblast stage, which can undergo limited granulocytic maturation on exposure to Me₂SO and limited monocytic maturation following treatment with TPA (18). However, uninduced or induced HL60 cells do not acquire secondary granules (19) and do not express mRNAs encoding other neutrophil secondary granule proteins, namely transcobalamin 1 (21), lactoferrin (20), and neutrophil collagenase (7). HNG mRNA expression was examined in CGL cells, peripheral neutrophils, and in HL60

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by National Institutes of Grant Health HD-22297 via the Pediatric Scientist Development Program.

¶ Supported by National Institutes of Health Grant GM-27939.

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¹ The abbreviations used are: MMP, matrix metalloproteinases; HNG, human neutrophil gelatinase (92-kDa type IV collagenase); CGL, chronic granulocytic leukemia; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; Me₂SO, dimethyl sulfoxide; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); kb, kilobase(s); PIPES, 1,4-piperazinediethanesulfonic acid.

cells, to assess whether it parallels that of other secondary granule protein genes.

MATERIALS AND METHODS

Restriction enzymes, T4 polynucleotide kinase, avian myeloblastosis virus reverse transcriptase, and S1 nuclease were from New England Biolabs (Beverly, MA); PCR kit was from Perkin-Elmer-Cetus; random-primed labeling kit and m⁷GpppG capping analog were from Boehringer Mannheim; DNA sequencing kit was from U. S. Biochemicals (Cleveland, OH); *in vitro* transcription and translation kits were from Promega (Madison, WI); L-[³⁵S]methionine, [³²P]ATP, and [³²P]dCTP were from Amersham Corp.; Enlightening[®] was from Du Pont-New England Nuclear.

Isolation of HNG Clones from CGL cDNA Library—The construction of the CGL cDNA library in Lambda-Zap II (Stratagene, La Jolla, CA) has been previously described (21). An antisense primer, CTGGGACCCCGCACCGTGG, based on the highly conserved cysteine switch region of the HT1080 gelatinase sequence (corresponding to the amino acids PRCGVPD, Ref. 8), and a sense oligonucleotide corresponding to the phage arm T3 sequence, were employed in a standard PCR (22), with CGL cDNA library as template. This yielded a distinct PCR product (Fig. 1), which was subcloned. Several positive subclones were sequenced and shown to be identical to the first 330 bp encoding HT1080 gelatinase. The PCR product was then used to screen the CGL cDNA library, as previously described (21). The positive clone with the largest insert (2.3 kb) was sequenced completely by the dideoxy method (23).

Isolation of Peripheral Neutrophil mRNA—Peripheral blood from a normal volunteer was anticoagulated with citrate and sedimented with an equal volume of 3% dextran T-500 (Pharmacia LKB Biotechnology Inc.) in 0.9% NaCl, at room temperature for 1 h (29). The buffy coat was centrifuged at 200 × *g* for 10 min at 4 °C and the pellets resuspended in ice-cold 0.9% NaCl. The suspension was layered over Ficoll-Paque (Pharmacia) and centrifuged at 1500 rpm for 30 min at room temperature. This maneuver separates the neutrophils and erythrocytes (in the pellet) from monocytes and lymphocytes (in the interface) and results in greater than 99% efficiency of separation of monocytes away from neutrophils (31). The pellet was resuspended in ice-cold sterile water for 30 s to lyse erythrocytes, brought up to 0.9% saline, and the neutrophils pelleted at 200 × *g* for 10 min at 4 °C. Lysis of remaining intact erythrocytes was repeated until a clear pellet was obtained, which was resuspended in guanidine isothiocyanate and spun over a cesium chloride gradient (30) to isolate total neutrophil RNA.

PCR for 92- and 72-kDa Gelatinases—The neutrophil RNA was reverse transcribed with random hexamer priming (27), and the resulting cDNA used as template in a standard PCR reaction. A sense oligonucleotide corresponding to the first 7 amino acids (ATGAGCCTCTGGCAGCCCTG) and an antisense primer based on the cysteine switch region of HNG were the Primers. This yielded a single distinct PCR product which was subcloned. Several subclones were sequenced and shown to be identical to the PCR product isolated from the CGL cDNA library.

The absence of monocytic sequences from the CGL cDNA library, CGL RNA, and neutrophil RNA was confirmed by taking advantage of the fact that monocytes and induced U937 cells secrete an additional 72-kDa gelatinase, which is absent from neutrophils (28). Total RNA from CGL cells, neutrophils, and TPA-induced U937 cells was reverse transcribed with random hexamer priming (27), and the resultant cDNAs used as template for two separate sets of PCRs. For the first set, the sense oligonucleotide corresponded to the first 7 amino acids, and the antisense to amino acids 42–48, of the 92-kDa gelatinase. For the second set of PCRs, the following primers based on the 72-kDa monocytic gelatinase sequence (2) were designed: a sense primer CTGCTGAGCCACGCCGCCGCC corresponding to amino acids 23–30, and an antisense primer GCGAGGGAAGAAGTTGTAGTTGCC corresponding to amino acids 111–118. The sequences encoded for by these primers are specific for the 72-kDa gelatinase and are absent from the 92-kDa enzyme.

In Vitro Translation of HNG Protein—A cDNA clone encoding HNG proenzyme was linearized and capped mRNA transcribed *in vitro* as described in the Promega protocols and applications guide (second edition, 1991). Transcription products were visualized by gel electrophoresis, quantitated by spectrophotometry, and 250 ng of mRNA translated in a 25-μl rabbit reticulocyte lysate reaction supplemented with [³⁵S]methionine. Translation products were immunoprecipitated with rabbit anti-HNG antibody, and products before

and after immunoprecipitation were analyzed by SDS-PAGE, followed by enhanced fluorography of the dried gel as previously described (7).

Primer Extension Studies—In order to determine the HNG transcript initiation site, three antisense oligonucleotides were synthesized: CCCAGCACCAGGAGCACC corresponding to nucleotides 46–63, CCAGGGAAGAGCACAAG corresponding to nucleotides 101–117, and ATTCCTCTGCCAGCTGCC corresponding to nucleotides 133–161 of the HNG cDNA. These were end-labeled, heated with 10 μg of total RNA from CGL and HL60 (negative control) cells in the presence of 0.1 mM EDTA at 65 °C for 10 min, cooled, annealed in 50 mM Tris, 6 mM MgCl₂, 40 mM KCl, 10 mM dithiothreitol at 65 °C for 15 min, and slowly cooled to room temperature over 3 h. dNTPs were then added to 2 mM and primer extension achieved with AMV reverse transcriptase for 10 min at room temperature, 10 min at 37 °C, and 60 min at 42 °C. The reactions were stopped with EDTA, the products digested with RNase, and analyzed by PAGE.

HNG Genomic Cloning and S1 Analysis—The 330-bp PCR product (Fig. 1) was used to screen the Maniatis human genomic library in Charon 4A, by previously described methods (24). The positive clone containing the largest insert (6.9 kb) was mapped and partially sequenced and shown to be identical to that of the 92-kDa human gelatinase gene recently reported (25). A DNA fragment containing the first exon of the 92-kDa gelatinase was end-labeled, and hybridized overnight in hybridization buffer (80% formamide, 1 mM EDTA, 0.4 M NaCl, 40 mM PIPES, pH 6.4) at 45 °C with 10 μg of total RNA from CGL or HL60 cells. Hybrids were digested with S1 nuclease and analyzed by PAGE.

RESULTS

Isolation, Confirmation, and Characterization of HNG cDNA Clone

An antisense oligonucleotide based on the conserved cysteine switch region of HT1080 gelatinase and a sense primer based on the phage T3 sequence were used in a PCR with CGL cDNA library as template. The distinct PCR product (Fig. 1) was subcloned, sequenced, and shown to have a nucleotide sequence identical to the first 330 bp of HT1080 gelatinase cDNA. Since monocytes also produce a 92-kDa gelatinase (28), and monocytic sequences may theoretically be present as contaminants in the CGL cDNA library, we verified the HNG cDNA sequence by isolation of an identical PCR product from a purified neutrophil RNA preparation. The neutrophil RNA was reverse transcribed and PCR performed with a sense primer based on the first 7 amino acids

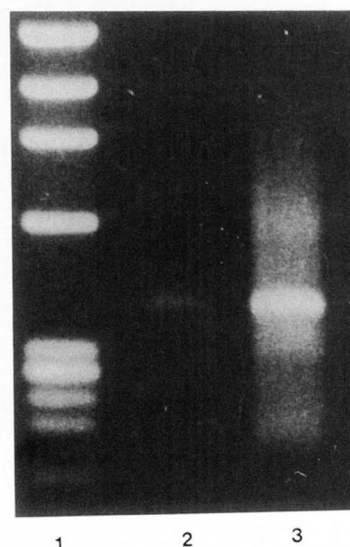


FIG. 1. Generation of PCR probe from CGL cDNA library. An antisense primer based on the cysteine switch of HT1080 gelatinase and a sense primer based on the phage arm T3 were used in a PCR with CGL cDNA library as template. Lane 1 is ϕ x174/HaeIII marker, lane 2 shows the PCR product (arrow), and lane 3 the results of reamplifying the product in lane 2. Similar results were obtained when reverse-transcribed neutrophil RNA was used as template.

of HT1080 gelatinase sequence and the same antisense primer as above. This yielded a distinct PCR product, the nucleotide sequence of which exactly matched that of the PCR product obtained from the CGL cDNA library. Based on this confirmation of the HNG sequence, and the exclusion of contaminating monocytic sequences (see below), the 330-bp PCR product was used to screen the CGL cDNA library. This yielded a 2.3-kb full-length HNG cDNA insert. Sequence analysis (not shown) revealed that the cDNA encoding HNG is identical to that of HT1080 gelatinase (8), with the following exceptions: there were an additional 15-bp of 3'-untranslated sequence in HNG, a base change at nucleotide position 855 from G to A (which alters the amino acid residue at position 179 from Arg in HT1080 to Gln in HNG), and a base change at position 1963 from C to G (which changes Ser at position 548 to Arg in HNG). Examination of the cDNA-derived amino acid sequence revealed that, like other MMPs, HNG has the requisite cysteine switch area (PRCGVPD), putative zinc-binding site (HEXXH), and characteristic domain structure.

Exclusion of Monocytic Sequences—The absence of monocytic sequences from the CGL cDNA library, CGL RNA, and neutrophil RNA was confirmed by taking advantage of the fact that monocytes and induced U937 cells secrete an additional 72-kDa gelatinase, which is absent from neutrophils (28). Total RNA from CGL cells, neutrophils, and induced U937 cells were reverse transcribed and used for two separate sets of PCRs. For the first set, the sense oligonucleotide corresponded to the first 7 amino acids, and the antisense to amino acids 42–48, of the 92-kDa gelatinase. This yielded distinct PCR products of the predicted 144-bp size (Fig. 2, top panel) from the CGL cDNA library, and reverse-transcribed CGL, neutrophil, and induced U937 cell RNA, but not from the negative water control. For the second set of PCRs, we used primer sequences which are specific for the 72-kDa gelatinase and are absent from the 92-kDa enzyme. Therefore, only those templates containing monocytic sequences should yield products amplified by these primers; accordingly, we observed a distinct PCR product of the predicted size (285 bp) only from induced U937 cell cDNA (Fig. 2, bottom panel). This PCR product was subcloned, sequenced, and shown to encode the monocytic 72-kDa gelatinase (2). The absence of

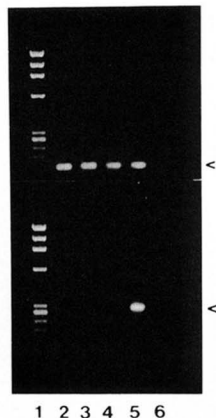


FIG. 2. Exclusion of monocytic sequences. cDNAs reverse transcribed from various RNAs were subjected to PCR with primers specific for the 92-kDa (top panel) or 72-kDa (bottom panel) gelatinase. Lane 1 is ϕ x174/HaeIII marker. Subsequent lanes show the products of amplifying CGL cDNA library (lane 2), CGL RNA (lane 3), neutrophil RNA (lane 4), induced U937 cell RNA (lane 5), and negative water control (lane 6). The 72-kDa monocytic gelatinase sequences were absent from CGL cDNA library, CGL RNA, and neutrophil RNA (bottom arrow).

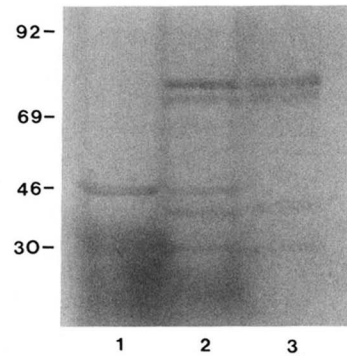


FIG. 3. *In vitro* translation of cDNA-derived HNG propeptide mRNA. Lane 1 shows translation products in the absence of added RNA; lane 2 shows the products with HNG RNA; lane 3 shows the products of immunoprecipitation of lane 2 with anti-HNG antibody. Numbers to the left denote molecular masses in kDa. Arrows to the right represent translation products recognized by the antibody.

a PCR product from the CGL cDNA library and from CGL and neutrophil RNAs indicates that these preparations are free from contaminating monocytic sequences. This absence of monocytic sequences from CGL RNA was further verified by Northern analysis. Both CGL and induced U937 cells were shown to express the 92-kDa HNG. However, only the U937 cells expressed the 72-kDa gelatinase mRNA, when probed with the product from the second set of PCRs mentioned above (data not shown).

***In Vitro* Translation of HNG Protein**—*In vitro* translation of mRNA synthesized from a cDNA encoding HNG proenzyme (from Ala at residue 20 to Asp at position 707), followed by immunoprecipitation with rabbit anti-HNG antibody, yielded a major protein product which corresponded to the predicted size of 78 kDa (Fig. 3). Three other translation products which were also recognized by the antibody migrated at approximately 65, 35, and 30 kDa. The 65-kDa product has a size appropriate for autolytically activated HNG, while the smaller two products are of the sizes expected for autolytically degraded HNG species. Thus, the translation products display the autolytic properties characteristic of MMPs. The discrepancy in size between the major *in vitro* translation product and the 92-kDa value observed for the secreted full-length protein is due to the absence of the signal peptide and inefficiency of glycosylation in the *in vitro* translation product. It is notable that all three potential *N*-linked glycosylation sites detected in the predicted amino acid sequence of the HT1080 gelatinase (8) are also present in the HNG sequence. The fact that native HNG exhibits the same apparent molecular mass of 92 kDa observed for the gelatinases produced by other cells (8) also suggests that the glycosylation patterns are very similar.

Determination of HNG mRNA Initiation Site—Both primer extension and S1 analysis showed a predicted mRNA initiation site corresponding to the first nucleotide in the HNG cDNA sequence, 19 bp 5' to the initiator methionine (Fig. 4). This agrees with the predicted mRNA initiation site for the gelatinase transcript in HT1080 cells, as determined by primer extension (25).

Northern Analysis—The 330-bp PCR product was used to probe total RNA from CGL cells and yielded a single mRNA species of 2.8 kb for HNG (Fig. 5). CGL cells did not express the 72-kDa gelatinase, indicating the absence of contaminating monocytic sequences from this preparation (not shown). Previous studies with the HT1080 gelatinase cDNA have shown the expression of a 2.8-kb message in transformed lung fibroblasts, induced HT1080 cells, and induced U937 cells (8).

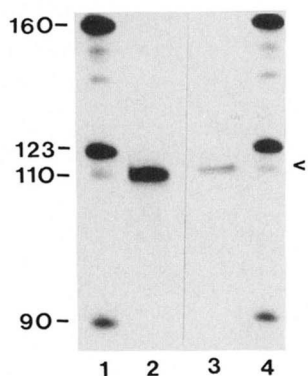


FIG. 4. Determination of HNG mRNA initiation site. Lanes 1 and 4 are molecular weight markers, as indicated on the left. Both S1 analysis (lane 2) and primer extension (lane 3) using a primer corresponding to nucleotides 100-117, predicted an mRNA start site 19-bp 5' to the initiator methionine. Similar predictions were obtained when two other oligonucleotides were used (not shown).

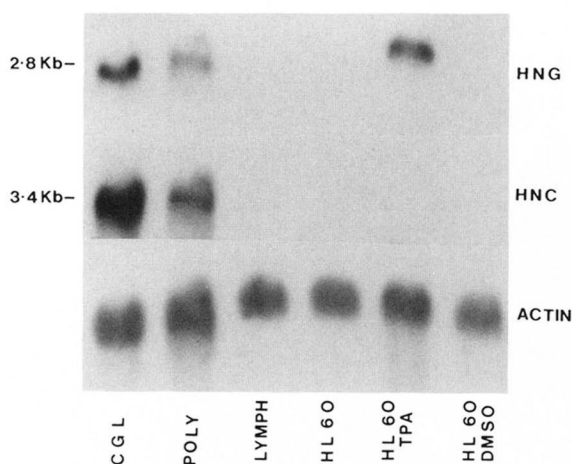


FIG. 5. Northern blot analysis of HNG expression. HNG was expressed as a 2.8-kb mRNA species in CGL, polymorphs (POLY), and HL60 cells induced to monocytic differentiation with TPA (HL60 TPA), and not in lymphocytes (LYMPH), uninduced HL60 cells, or in Me₂SO-induced HL60 cells (HL60 DMSO), as shown in the top panel. In contrast, human neutrophil collagenase (HNC, middle panel) was not expressed in HL60 TPA. The bottom panel shows the same blot probed with actin, as a control for RNA loading.

Interestingly, mature peripheral neutrophils from normal subjects were also found to express HNG. The neutrophil RNA used for Northern analysis was previously shown to be free of monocytic sequences by PCR (Fig. 2). HNG mRNA was absent from several normal tissues examined, including fibroblasts, stomach, colon, pancreas, kidney, ovary, breast, and brain (not shown).

We also examined HNG expression in uninduced and induced HL60 cells to define the identity of HNG as a neutrophil secondary granule protein gene. As has been previously noted for lactoferrin (20), transcobalamin I (21), and neutrophil collagenase (7), HL60 cells in the uninduced state and induced to a neutrophil phenotype with Me₂SO did not express HNG mRNA. However, HNG was detected in HL60 cells induced to monocyte/macrophage differentiation by exposure to TPA (Fig. 5). This is a unique feature of HNG, since other secondary granule genes are not expressed in TPA-induced HL60 cells and are not expressed in mature human macrophages or monocytes.

DISCUSSION

Neutrophils synthesize and store intracellularly a 92-kDa gelatinase, the primary structure and expression of which

were examined. With a CGL cDNA library and reverse-transcribed neutrophil RNA as templates, PCR was used to isolate identical probes encoding for the first 330 bp of neutrophil gelatinase. The cDNA library was then screened with the probe. Cell-free translation of the cDNA-derived HNG mRNA yielded products which were recognized by anti-HNG antibody. Furthermore, the products were of sizes appropriate for HNG proenzyme, autolytically activated HNG, and autolytically degraded species. This fragmentation pattern is characteristic of MMPs (7). The cell-free translation system is therefore well suited for investigating the primary structural basis for the autolytic functions of HNG.

The HNG cDNA sequence was identical to that of a 92-kDa gelatinase isolated from TPA-induced HT1080 cells (8), except for 2 single base pair changes which probably represent genetic polymorphisms. In addition, both primer extension and S1 analysis revealed that the transcription initiation site for gelatinase mRNA in CGL cells is the same as that recently reported in induced HT1080 cells (25). Thus, although neutrophil interstitial collagenase has been shown to be encoded for by a gene distinct from fibroblast interstitial collagenase (7), the gelatinase gene appears to be identical in both neutrophils (which store the enzyme in secondary granules) and TPA-induced fibrosarcoma cells (which constitutively secrete the enzyme). Complete conservation of the 92-kDa gelatinase between these and several other cell types (macrophages/monocytes, keratinocytes, induced fibrosarcoma and monocytic leukemia cell lines, and transformed fibroblasts) is perhaps indicative of a relatively primitive or fundamental function for this enzyme in humans. In addition, it is intriguing to speculate that distinct intracellular mechanisms may dictate the intracellular storage of this enzyme by neutrophils and its constitutive secretion by other cells.

Since monocytes also secrete a 92-kDa gelatinase (28), we have employed several methods to exclude contamination of the CGL cDNA library, CGL RNA, and neutrophil RNA, by monocytic sequences. First, we have shown by reverse transcription and PCR that only TPA-induced U937 cells yielded a product encoding for the 72-kDa monocytic gelatinase (as previously described, Refs. 2 AND 28); this PCR product was absent from the CGL cDNA library, CGL RNA, and neutrophil RNA. Second, we have shown by Northern analysis that the CGL RNA does not express the 72-kDa monocytic gelatinase. Third, we have isolated a partial cDNA by reverse transcription and PCR of the purified uncontaminated neutrophil RNA, the sequence of which exactly matches that of the HNG cDNA isolated from uncontaminated CGL cDNA library.

Expression of HNG mRNA was absent from several normal tissues examined but was detected in mature peripheral neutrophils from normal adults, although at a lower abundance than in CGL cells (Fig. 5). Similar results were obtained for another neutrophil secondary granule gene, namely collagenase. These observations are consistent with the hypothesis that although neutrophil secondary granule proteins are a hallmark of the developing neutrophil, they continue to be expressed by the mature neutrophil. Whether the decreased abundance of these messages in the mature cell is related to diminished transcription rates or to increased mRNA degradation is not known.

Northern blot analysis also revealed the presence of HNG mRNA in HL60 cells induced to monocytic maturation with TPA. This is in contrast to the absent expression of HNG and of all other neutrophil secondary granule protein genes in HL60 cells induced along the neutrophilic pathway with Me₂SO. This finding raises the possibility that the HNG gene

may be subject to differential control pathways in two related but distinct hematopoietic lineages. The induction of HNG expression in HL60 cells by TPA suggests that monocytic expression may also be related to TPA response elements which have been identified in the putative promoter region of the HNG gene (25). Potential regulatory factors dictating the expression of HNG and other coordinately regulated secondary granule protein genes in developing neutrophils remain to be determined. Also, the molecular defect leading to the absence of neutrophil secondary granule genes in Me₂SO-treated HL60 cells remains to be elucidated.

Acknowledgment—We thank Mariana Dioszegi for expert technical assistance.

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