Journal of Cellular Biochemistry 59:431–442 (1995)

# Molecular Cloning of a Novel Myeloid Granule Protein

**Lynn C. Moscinski and Bobbye Hill**

Department of Pathology, H. Lee Moffitt Cancer Center at the University of South Florida, Tampa, Florida 33612

**Abstract** Granulocytes are recognized by the presence of granules, including primary (azurophilic) and secondary types. Each granule type contains distinct and characteristic families of enzymes. We have screened a murine bone marrow cDNA library to obtain a series of sequences corresponding to mRNAs which are both myeloid-specific and appear to be expressed only in immature bone marrow cells. A 1,160 bp sequence (B9) has been isolated, which shows restricted expression in murine bone marrow, with the highest levels in cultures enriched for promyelocytes. Translation yields a single open reading frame of 167 amino acids and a calculated MW of 19.33 kd. A single potential N-glycosylation site is present. Evaluation of the amino terminal sequence shows 2 polar amino acids flanking a hydrophobic region, suggesting a signal sequence and the possibility of post-translational modification. An extensive search of the protein data base reveals 30% identity over 90 amino acids with porcine cathelin, a cystatin-like cysteine proteinase inhibitor. This sequence identity includes conservation of the 4 cysteine residues noted in all members of the cystatin superfamily. In an attempt to further characterize this novel sequence, a polyclonal antiserum was prepared by immunization with a 20 amino acid synthetic peptide corresponding to a unique portion of the carboxy terminus. Immunoelectron microscopy localized B9 to neutrophilic granules. We have identified a novel myeloid-specific granule protein related to porcine cathelin, but showing important structural differences. This may represent the first isolated member of a new cystatin family. More importantly, the small size of the B9 gene and its tight pattern of early expression make B9 an excellent reporter molecule for the study of new factors important in myeloid differentiation. 

**Key words:** myeloid differentiation, promyelocyte, bone marrow, murine, cDNA

Hematopoietic stem cells are believed to give rise to committed progenitor cells within the bone marrow and subsequently undergo a program of differentiation which results in the production of mature blood elements [Cronkite, 1988]. These elements include platelets, red cells, and myeloid cells, the latter of which consists of both granulocytes and monocytes. Granulocytic differentiation is recognized by morphologic criteria after staining preparations of bone marrow with Wright Giemsa or other dyes. The earliest recognizable granulocytic precursor is a promyelocyte, identified by its complement of large azurophilic (primary) granules, as well as its immature nucleus. As maturation progresses, the granulocytic cell cytoplasm becomes pink-orange in color as it is filled by the production of large numbers of minute (secondary) granules. Both subtypes of granules contain distinct and characteristic families of enzymes.

Received March 15, 1995; accepted April 11, 1995.

Address reprint requests to Lynn C. Moscinski, MD, Department of Pathology, H. Lee Moffitt Cancer Center and Research Institute, 12902 Magnolia Drive, Tampa, FL 33612.



Molecular biology has made it possible to clone and characterize several myeloid genes. They can be divided into several distinct groups, broadly classified as coding for ubiquitous ("housekeeping") proteins and granulocyte specific proteins [Lubbert et al., 1991]. Many ubiquitously expressed proteins are necessary for cellular proliferation and survival. Myeloid specific proteins include those involved in differentiation, cellular recognition, and cell-cell interaction, as well as the definition of a functional cell type.

The majority of myeloid-specific genes which have been cloned and characterized correspond to surface or cytosolic proteins present either late in neutrophil differentiation or throughout the entire myeloid maturational scheme. These include NADPH oxidase cytochrome b heavy and light chains [Royer-Pokora et al., 1986; Barker et al., 1988; Parkos et al., 1988], NADPH oxidase cytosolic p47 and p67 proteins [Volpp et al., 1989; Leto et al., 1990], transcobalamin I [Johnston et al., 1989], lysozyme [Castanon et al., 1988; Chung et al., 1988], CD16 [Peltz et al., 1989], CD13 [Look et al., 1989], CD33 [Sim-

mons and Seed, 1988; Peiper et al., 1988], CD14 [Goyert et al., 1988; Simmons et al., 1989; Ferrero and Goyert, 1988], CD11b [Hickstein et al., 1989; Arnaout et al., 1988], and guinea pig neutrophil cationic peptide [Nagaoka et al., 1992]. Only a few examples of genes expressed only by immature myeloid cells (promyelocytes) have been characterized in any detail, and they represent constituents of myeloid granules. These are myeloperoxidase [Tobler et al., 1988; Sagoh and Yamada, 1988], cathepsin G [Salvesen et al., 1987], neutrophil elastase [Takahashi et al., 1988; Fouret et al., 1989], defensin [Wiedemann et al., 1989], and myeloblastin [Bories et al., 1989]. Clearly, these represent only a minority of the proteins found in the granules of developing neutrophils.

We have screened a murine myeloid cDNA library by differential hybridization [Moscinski and Prystowsky, 1990] to obtain a series of myeloid clones corresponding to mRNAs which are both myeloid specific and differentiation stage specific. One clone, B9, has been identified as a constituent of neutrophilic granules, and has sequence homology to members of the cystatin superfamily of cysteine proteinase inhibitors. It is expressed by immature myeloid cells of normal mouse bone marrow, with the highest levels of mRNA detected in cultures containing predominantly promyelocytes. Further analysis of the genomic structure of B9 will hopefully lead to a better understanding of the mechanisms involved in tissue and differentiation stage specific gene expression.

## MATERIALS AND METHODS

### Source of Bone Marrow and Culture

Bone marrow was expressed from bilateral tibias and femurs of 10-week-old Balb/c mice using Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) fetal calf serum, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and additional amino acids as previously described [Jaffe et al., 1988]. For culture, bone marrow was semipurified by density centrifugation over Ficoll-Hypaque (1.065) and plated in bone marrow medium: alpha medium supplemented with 20% (vol/vol) fetal calf serum, 100 pM sodium selenite, 100 U/ml of penicillin, and 100 μg/ml streptomycin, and stimulated with 5 U/ml recombinant murine GM-CSF (Amgen, Thousand Oaks, CA). Cells were harvested on sequential days by vigorous pipetting. Cultures were monitored daily by morphological examination after preparation of cytospins and staining with Wright Giemsa. Cells obtained on day 3 after GM-CSF stimulation consisted predominantly of promyelocytes, while those on days 7–14 were predominantly segmented neutrophils with admixed adherent macrophages [Jaffe et al., 1988].

### RNA Preparation and Northern Blotting

Bone marrow cells were harvested and pelleted by centrifugation. RNA was isolated by the guanidinium isothiocyanate-cesium chloride method [Chirgwin et al., 1979] and quantitated by absorbance at 260 nm. Ten micrograms of each sample was size fractionated by electrophoresis on a 1% agarose gel containing 6% formaldehyde and 200 ng/ml ethidium bromide in 20 mM MOPS, 5 mM sodium acetate, and 1 mM EDTA. Following electrophoresis, RNA was visualized with UV illumination to determine the location of the 28S and 18S ribosomal bands, to assess the integrity of the RNA, and to verify that equal amounts of RNA had been loaded per lane. RNA was transferred to Hybond N nylon membrane and hybridized according to the manufacturer's specification. Double stranded plasmid (p-Bluescript) containing the cloned B9 insert was labeled with (32)P by random priming, and used as a probe. A 1.5 kb human β-actin cDNA was used as a hybridization control [Gunning et al., 1983] and to assure equal loading of all lanes.

### DNA Sequence Analysis

Six cross-hybridizing and overlapping cDNA clones, isolated from the original differential screening of a murine bone marrow cDNA library [Moscinski and Prystowsky, 1990], were sequenced bidirectionally and in their entirety using a series of synthetic oligonucleotides as primers. All oligonucleotides were prepared in the University of Florida (Gainesville, FL) DNA synthesis core facility, and were shipped as lyophilized pellets. Sequencing was performed according to the method of Sanger [Sanger et al., 1977], using Sequenase enzyme (United States Biochemical, Cleveland, OH). Gels were 6% acrylamide and 7 M urea in Tris-borate buffer.

Computer assisted sequence analysis utilized programs available on the Genetics Computer Group Sequence Analysis Software Package (University of Wisconsin, Madison, WI), available through the University of Florida Computer Network. An open reading frame was es-

tablished using FRAMES [Devereux et al., 1984], protein translation was performed using TRANSLATE [Devereux et al., 1984], protein secondary structure was predicted with PEPTIDE STRUCTURE [Jameson and Wolf, 1988] and plotted graphically with PLOT STRUCTURE [Jameson and Wolf, 1988]. Both protein and nucleic acids databases were searched for similarities to B9 using the BLAST program [Benson et al., 1990].

The complete nucleotide sequence for B9 has been submitted to GenBank (L37297).

### Preparation of a Polyclonal Antiserum in Rabbits

The translated amino acid sequence of B9 was examined to detect potential peptide fragments suitable for production of polyclonal antisera. The carboxy one-third of the protein showed no homology to any sequence in the protein or nucleic acid databases, such that production of a specific, potentially non-cross reactive antibody was more likely. Structural analysis suggested the presence of a helical segment containing 8 charged amino acids beginning at the terminal cysteine. A 20 amino acid synthetic peptide was commercially synthesized corresponding to: CSREDTQETSFNDKQDVSEK. The length of the peptide was chosen to represent complete turns of the putative helical structure, thus theoretically stabilizing the fragment into a form resembling its native state in vivo. A commercially prepared (ImmunoDynamics, Inc., La Jolla, CA) polyclonal antibody was produced after conjugation to KLH and injection into rabbits with Freund's adjuvant. Specificity was monitored by ELISA. Antiserum from the final bleed was affinity purified by adsorption to a matrix containing bound synthetic peptide and subsequently eluted.

### Protein Isolation and Western Blot Analysis

Murine liver, spleen, and bone marrow were isolated fresh and lysed in Triton X-100. The lysates were cleared by centrifugation and stored frozen at −70°C until use. Total protein was quantitated colorimetrically after formation of a pyrogallol red-molybdate complex (Biotrol). Fifty micrograms total protein was loaded in each lane of a denaturing 15% polyacrylamide gel after heat denaturation at 95°C for 5 min. Five micrograms synthetic B9 peptide was used as a positive control for hybridization. Following separation, proteins were transferred to polyvinyldifluorine (PVDF) membrane according to the manufacturer's specifications. Membranes were incubated with a 1:2,000 dilution of affinitypurified anti-B9 antiserum overnight at 4°C, and the blots developed using a chemiluminescent detection system (Amersham, Arlington Heights, IL). As a negative control, the blot was incubated with pre-immune serum at a 1:1,000 dilution, and re-developed.

### Immunohistochemistry

Cytospins were prepared from Ficoll-Hypaque semi-purified murine bone marrow and air dried. They were subsequently fixed in 50% anhydrous methanol/50% acetone (vol/vol) for 4 min and rinsed in phosphate buffered saline (PBS). Slides were incubated overnight at room temperature with anti-B9 antiserum at a concentration of 1:100. Detection was by an avidin/biotin alkaline phosphatase technique (Dako, Carpinteria, CA). Negative control antiserum was an irrelevant anti-human CD45RA.

### Immunoelectron Microscopy

Bone marrow aspirate was fixed in 2.5% (w/v) glutaraldehyde and embedded in epoxy resin. Ultrathin sections were cut and mounted on nickel grids. The grids were floated on a solution containing ovalbumin in phosphate-buffered saline (PBS) (Reagent C, E.Y. Laboratories, Inc., San Mateo, CA) for 10 min to block non-specific binding sites. Grids were transferred, without rinsing, onto a drop of anti-B9 which was diluted 1:50 with a solution containing bovine serum albumin, Triton X-100, and Tween 20 in PBS (Reagent E, E.Y. Laboratories). Anti-lysozyme (Zymed Labs, Inc., San Francisco, CA) at a concentration of 1:10 was used as a positive hybridization control, and pre-immune serum at a concentration of 1:10 was used as a negative control. Incubation proceeded overnight in a moist chamber at room temperature. The grids were then washed with PBS and again floated on a drop of PBS/ovalbumin for 5 min, followed by transfer, without rinsing, to a drop of protein A-gold stock solution (colloidal gold 15 nm, Reagent L, E.Y. Laboratories) diluted 1:10 with PBS. Incubation proceeded for 1 h in a moist chamber. The grids were washed with PBS and then with distilled water. They were allowed to air dry, and were stained with uranyl acetate followed by lead citrate [Roth, 1986; Bendayan et al., 1986]. All grids were examined with the use of a Philips (Mahwah, NJ) CM-10 transmission electron microscope.

## RESULTS

### B9 Represents a Novel Murine Myeloid Specific Gene

Screening of a murine promyelocyte library using differential hybridization has led to the identification of a novel cDNA, B9, which appears to be both bone marrow specific and differentiation stage specific [Moscinski and Prystowsky, 1990]. It is expressed on Northern blot (Fig. 1) in a manner similar to myeloperoxidase [Jaffe et al., 1988], with maximal RNA accumulation on day 3 of culture following stimulation with GM-CSF. This time point has been previously shown to correspond to cultures in which promyelocytes are the predominant cell type identified [Jaffe et al., 1988] (Fig. 2). B9 RNA rapidly decreases as granulocytic maturation ensues, and is undetectable after 5 days in culture, a time when segmented neutrophils and macrophages are the major constituents. Thus, B9 represents a myeloid specific gene which is tightly regulated during early myeloid differentiation.

Six cross-hybridizing and overlapping cDNA clones were sequenced bidirectionally and in their entirety using a series of synthetic oligonucleotides as primers. A single clone (clone B6) contained an apparent full-length insert corresponding to 1,160 bp (Fig. 3). A single long open reading frame was identified, corresponding to 167 amino acids and a calculated molecular weight of 19.33 kd. A single potential N-glycosylation site is present. The amino terminus contains 2 polar amino acids flanking a hydrophobic region, suggesting a signal sequence ($NH_2$-MAGWKTFVLVVALAVS) and the possibility of post-translational modification of this protein. The predicted isoelectric point is 5.23.

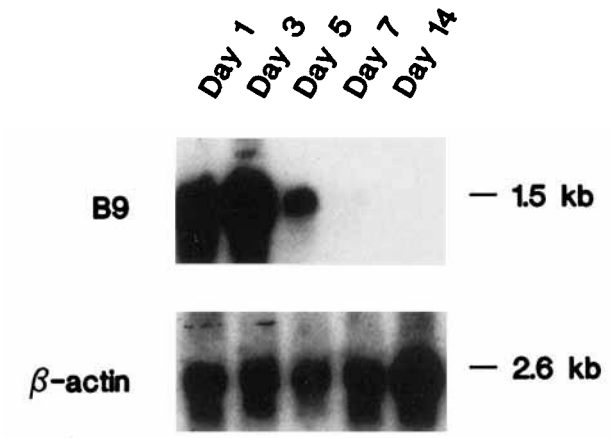


**Fig. 1.** *Northern blot analysis of clone B9 demonstrates regulated expression in murine bone marrow.* Ten micrograms of RNA were analyzed at sequential days following stimulation with GM-CSF. B9 is present in the relatively heterogeneous population present on day 1, but is up-regulated at day 3 when promyelocytes constitute greater than 50% of the recognizable cells. The signal for B9 is maximal at day 3 and then sharply declines by day 5. It is undetectable in cultures consisting predominantly of neutrophils and macrophages (days 7+). Equal loading of RNA was judged by comparison to actin hybridization.

An extensive search of both protein and nucleic acid databases showed no identity to any previous published sequence. However, the central region of the new protein B9 showed 30% identity over 90 amino acids with porcine cathelin, including conservation of the characteristic 4 cysteine residues noted in all members of the cystatin superfamily [Muller-Esterl et al., 1985], as well as the presence of 16 amino acids between the third and fourth cysteines (Fig. 4). Of interest, cathelin, like our novel protein B9 but unlike all other cystatin family members, lacks methionine. However, unlike cathelin, B9 contains an amino-terminal signal sequence motif, a non-homologous and extended carboxy terminus, and an Arg-Arg-Arg sequence in the middle of the protein. We postulate that B9 may represent a myeloid specific protein related to porcine cathelin, but showing important structural differences. The putative amino acid sequence, hydrophilic nature, and the presence of a possible signal peptide suggest that it may be a secreted protein.

### Western Blot Analysis Confirms the Predicted Structure of B9

Western blot analysis (Fig. 5) was used to verify that B9 coded for a bone marrow specific protein, and to determine its molecular weight. The major band noted in lanes containing liver lysate was visualized at lower concentrations in all lanes after longer exposure, and was identified in liver lysate lanes after rehybridizing with preimmune serum. This band co-migrates with ovalbumin, thus most likely representing a non-specific signal. Two distinct and unique bands were identified in murine bone marrow but not in any other tissue studied. The larger band of this doublet corresponds to the predicted molecular weight of 19.3 kd. The smaller band (approximately 17 kd) may be a result of post-translational modification of the protein, or more likely proteolytic degradation (a common finding during the isolation of neutrophil proteins). These results support the predicted amino acid se-

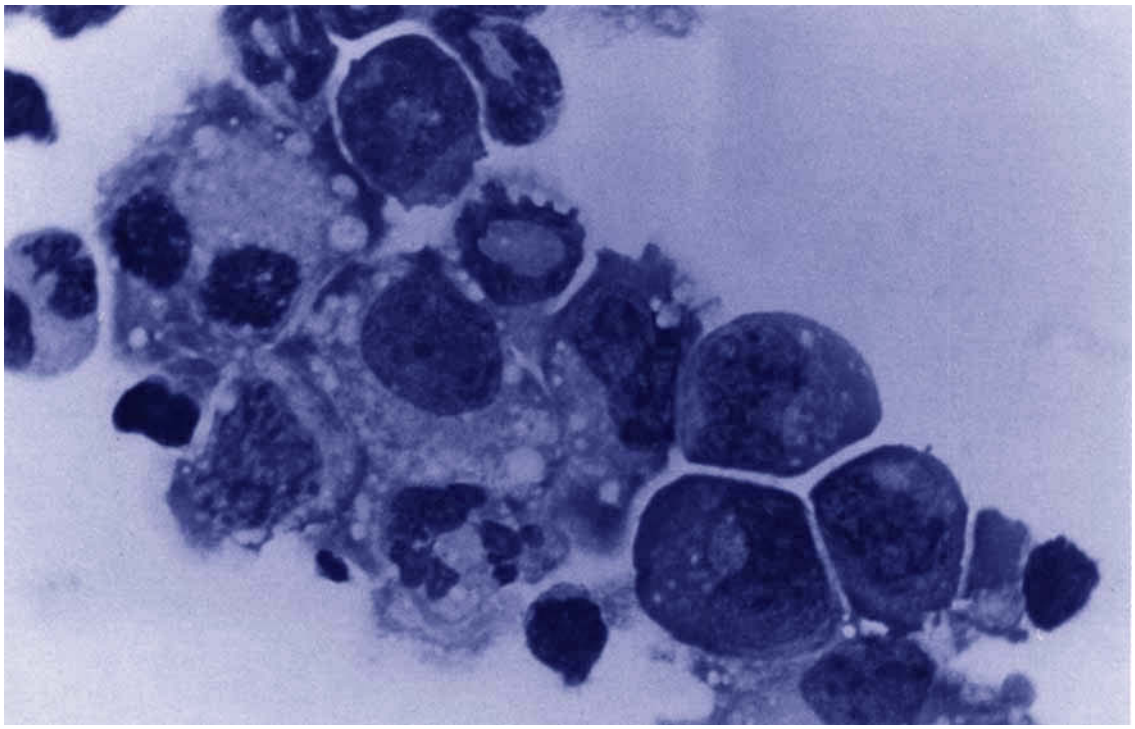

**Fig. 2.** Wright Giemsa stained cytospin of cultures after 3 days incubation with GM-CSF shows enrichment for promyelocytes, which are characterized by primitive chromatin and the presence of large azurophilic granules within the cytoplasm. Secondary granules within cells from this mouse strain are much smaller, and fail to impart any detectable pink-orange color to the cytoplasm; thus it appears grey. Original magnification, ×500.

quence of clone B9 and verify tissue specific gene expression.

### B9 Localizes to Neutrophilic Granules

Immunohistochemistry was used to determine which bone marrow cell types expressed B9 protein. Cytospins of partially purified murine bone marrow were stained with anti-B9 and evaluated. Diffuse cytoplasmic positivity was evident in band and segmented neutrophils, with distant Golgi positivity noted in less mature myeloid cells (Fig. 6). No staining was identified within the cytoplasm of megakaryocytes, erythroid cells, or lymphocytes. Too few monocytes were present for adequate evaluation. Basophils and eosinophils could not be reliably distinguished from neutrophils with hematoxylin counterstaining.

In order to evaluate positivity within bone marrow lineages and maturational stages, and to determine the subcellular localization of B9 protein, immunogold electron microscopy was performed. B9 was identified only in neutrophilic precursors, and was not seen in macrophages or eosinophils. As was noted by light microscopy, B9 was also absent in megakaryocytes, erythroid cells, and lymphocytes. Furthermore, B9 was localized to a population of pleomorphic cytoplasmic specific granules (Fig. 7A,B). When day 3 promyelocyte cultures were similarly evaluated, no staining was seen, although granules were very infrequent and poorly visualized, most likely due to the lack of osmium or other post-fixation (Fig. 7C). Staining intensity was suboptimal to evaluate whether Golgi or endoplasmic reticulum (ER) positivity existed in these more immature cells. Parallel experiments using formalin or alcohol fixatives demonstrated a decrease in B9 staining intensity following glutaraldehyde fixation, suggesting an effect of protein denaturation on the quality of staining intensity. However, only glutaraldehyde allowed reasonable visualization of the granule outlines in the absence of post-fixation with osmium. Fixation with 2% osmium tetroxide improves membrane resolution, but significantly ablates staining intensity with immunogold reagents while it increases nonspecific background [Moscinski et al., 1992]. When parallel grids from these cultures were stained for lyso-

```
        CACAGCTGGTAATACCAATGTGGTTTGAAGATTATGAGTGAACTGATGTGTGAGAGACA

          NH2-Met  Ala Gly  Leu Trp  Lys Thr  Phe Val  Leu Val  Val Ala
60            ATG GCA GGG CTG TGG AAG ACC TTT GTA TTG GTG GTG GCC

          Leu Ala  Val Val  Ser Cys  Glu Ala  Leu Arg  Gln Leu  Arg Tyr
99        TTG GCT GTG GTC TCC TGT GAG GCC CTT CGA CAA CTA AGA TAT

          Glu Glu  Ile Val  Asp Arg  Ala Ile  Glu Ala  Tyr Asn  Gln Gly
141       GAG GAG ATT GTT GAT AGA GCC ATA GAG GCA TAC AAC CAA GGG

          Arg Gln  Gly Arg  Pro Leu  Phe Arg  Leu Leu  Ser Ala  Thr Pro
183       CGG CAA GGA AGA CCC CTC TTC CGC CTG CTA AGT GCC ACT CCG

          Pro Ser  Ser Gln  Asn Pro  Ala Thr  Asn Ile  Pro Leu  Gln Phe
225       CCT TCT AGT CAG AAC CCT GCT ACC AAT ATC CCA CTC CAG TTC

          Arg Ile  Lys Glu  Thr Glu  Cys Thr  Ser Thr  Gln Glu  Arg Gln
267       AGG ATT AAA GAG ACA GAG TGT ACT TCC ACC CAG GAG AGA CAG

          Pro Lys  Asp Cys  Asp Phe  Leu Glu  Asn Gly  Glu Glu  Arg Asn
309       CCT AAA GAC TGC GAC TTC CTG GAG AAT GGG GAG GAG AGA AAT

          Cys Thr  Gly Lys  Phe Phe  Arg Arg  Arg Gln  Ser Thr  Ser Leu
351       TGC ACA GGG AAA TTC TTC AGA AGG CGG CAG TCA ACC TCC CTG

          Thr Leu  Thr Cys  Asp Arg  Asp Cys  Ser Arg  Glu Asp  Thr Gln
393       ACC TTG ACC TGC GAC AGG GAT TGC AGT CGA GAG GAT ACC CAA

          Glu Thr  Ser Phe  Asn Asp  Lys Gln  Asp Val  Ser Glu  Lys Glu
435       GAA ACC AGT TTT AAT GAT AAG CAA GAC GTC TCC GAA AAG GAA

          Lys Phe  Glu Asp  Val Pro  Pro His  Ile Arg  Asn Ile  Tyr Glu
477       AAG TTC GAA GAT GTG CCC CCT CAC ATC AGG AAC ATT TAT GAA

          Asp Ala  Lys Tyr  Asp Ile  Ile Gly  Asn Ile  Leu Lys  Asn Phe
519       GAT GCC AAG TAT GAT ATC ATC GGC AAC ATC CTG AAA AAT TTC

561   TAGGGCTGGAAAGAGGAGGGAGGTGCTCCCTGCATACTATGACCTCCTCTTTACCTCCACT
622   ACCCATCTCCCCCTGCTGCCTTCAGGATCTGCCCCTCCTTCCTGCCCTTCCCAGGAACACC
683   CCCTCTAGAGTAGCTCTAGCTCCTAAAACATCCATACCTTTGTCCATTTGCTTCCTTCTGC
744   TGGGCCTTCCTGCCTTACCCTCTATCTGAAACCCTTATTGATTCTTCAAGGCCCAAGTTCA
805   AAAGTCCCCTCCAGCGGGAAGCCTCCTCATTCTCCCAGAGCCAAAGTCCTGCCCACATCAG
866   TTCACTCATAATCTTCAAACCACATTGGTATTACCTGCTGTGTCCCCAGCCAGACAACCCT
927   GTATCTATTCACAGCTGGCTCCCGGGCCAGTTGCAGGTAGAATGAATATTTCAATGATGTG
988   TCCCGGAATCCTGGGAGGACAGAACCCTGTAGACTCCTGCTCTCTGCCTAGTCACTGTGAC
1049  ACCAAATGCCCCTTTACATACCCAGATCCTTAACGGGGATGTGGCAGGTGGGTGTGGTCAG
1110  ATCACCTTGTGAGGCCTATAAGAGAGGTTCAATAAAAATGCTTCTGAGATT
```

**Fig. 3.** The nucleotide sequence of the coding strand of B9 is shown, as well as an overlay of the translated amino acid sequence with the amino terminus labeled. The polyadenylation signal, AATAAA, is underlined. The four conserved cysteines, homologous to other cystatin family members, are shown in bold.

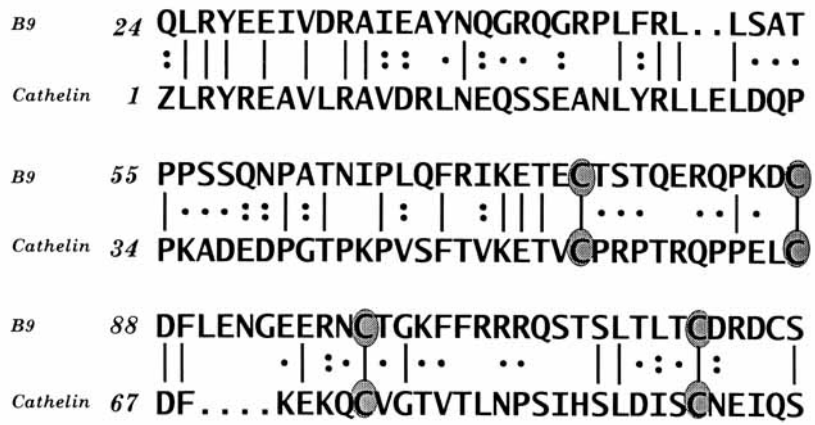


**Fig. 4.** Schematic representation illustrates limited sequence homology between murine B9 (**upper**) and porcine cathelin (**lower**). Thirty percent identity exists over 90 amino acids, including conservation of the characteristic 4 cysteine residues noted in all members of the cystatin superfamily and the presence of 16 amino acids between the third and fourth cysteines.

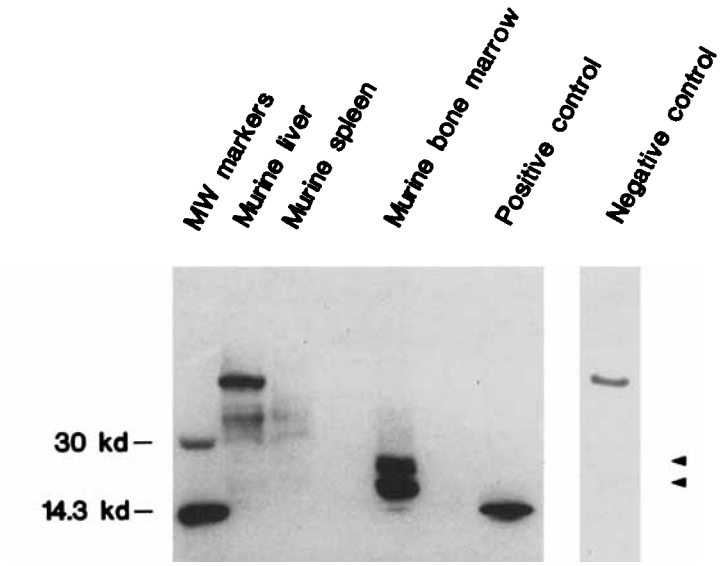


**Fig. 5.** Western blot analysis supports the predicted amino acid sequence of clone B9 and verifies tissue specific expression. Fifty micrograms total protein from murine bone marrow, spleen, and liver are loaded. The synthetic peptide which served as the B9 antigen for immunization is also loaded as a positive control for hybridization. A doublet is identified in murine bone marrow and not seen in any other tissue tested. The upper band corresponds to the predicted molecular weight of B9. The blot was stripped and re-hybridized with preimmune serum. A high molecular weight band remains detectable in liver lysates (negative control). This most likely represents ovalbumin and is a nonspecific signal.

zyme, a distribution of granule positivity similar to that of B9 was noted (Fig. 7D). Although some authors claim to detect neutrophil lysozyme only in primary granules [Mason et al., 1975], we and others [Moscinski et al., 1992; Asamer et al., 1971] have identified lysozyme protein in secondary granules of mature human neutrophils. Thus, B9 co-localizes with neutrophil lysozyme and corresponds to a novel secondary granule protein structurally related to a family of cysteine proteinase inhibitors. Whether B9 is also present in primary granules is unclear from this study. More intense staining and better post-fixation methods will be required to make this determination.

## DISCUSSION

The process of bone marrow differentiation is accompanied by the coordinate expression of numerous genes in a cell type specific and differentiation stage specific manner. Molecular biology has made it possible to clone and character-

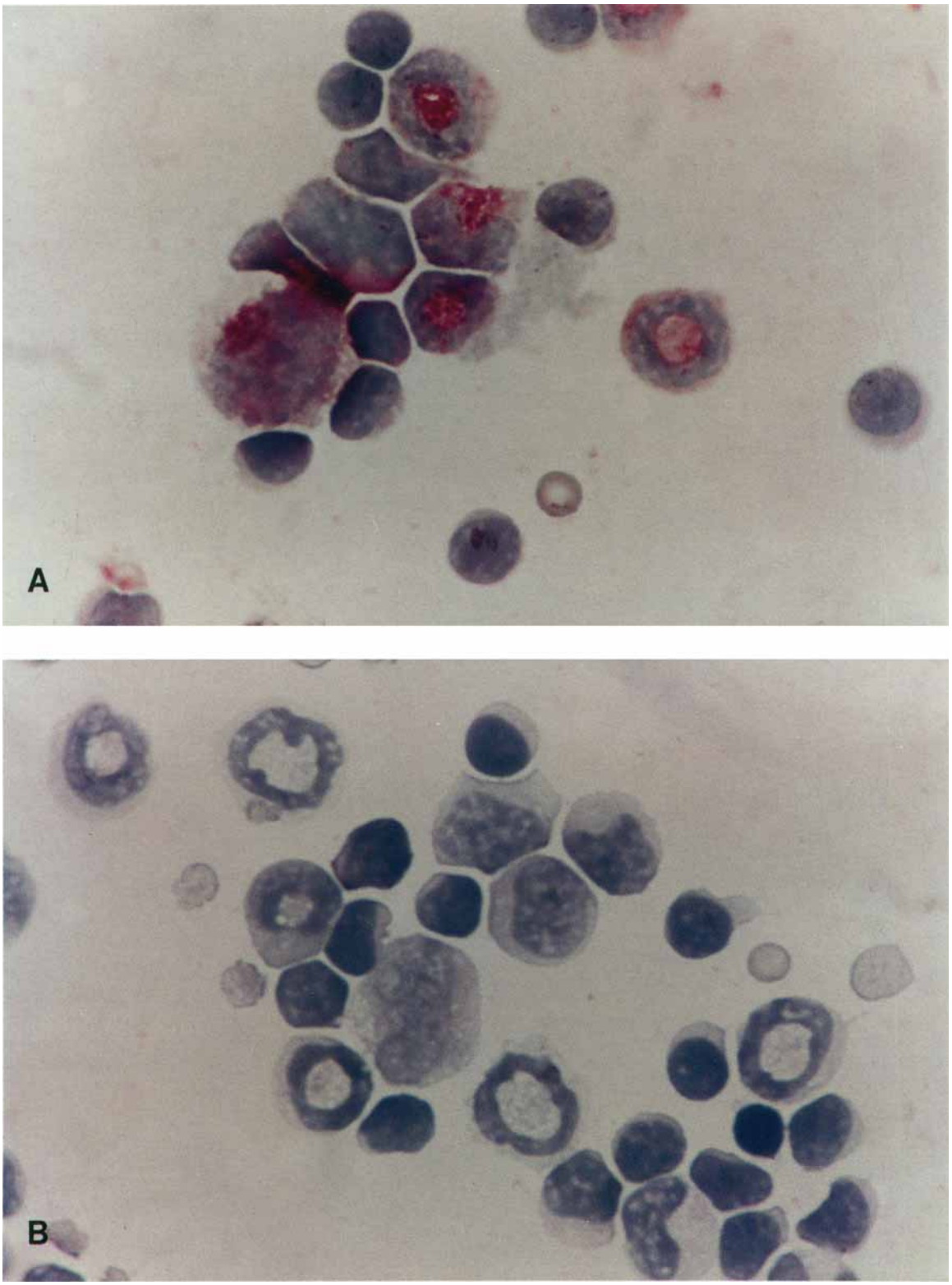


**Fig. 6.** Immunohistochemistry localizes B9 to the cytoplasm of granulocytes, with intense staining in segmented neutrophils and Golgi positivity in less mature myeloid cells (**A**). No staining is seen in lymphocytes or megakaryocytes. An irrelevant control, anti-human CD45A, serves as a negative background control (**B**) to exclude endogenous staining. Alkaline phosphatase and hematoxylin, original magnification ×500.

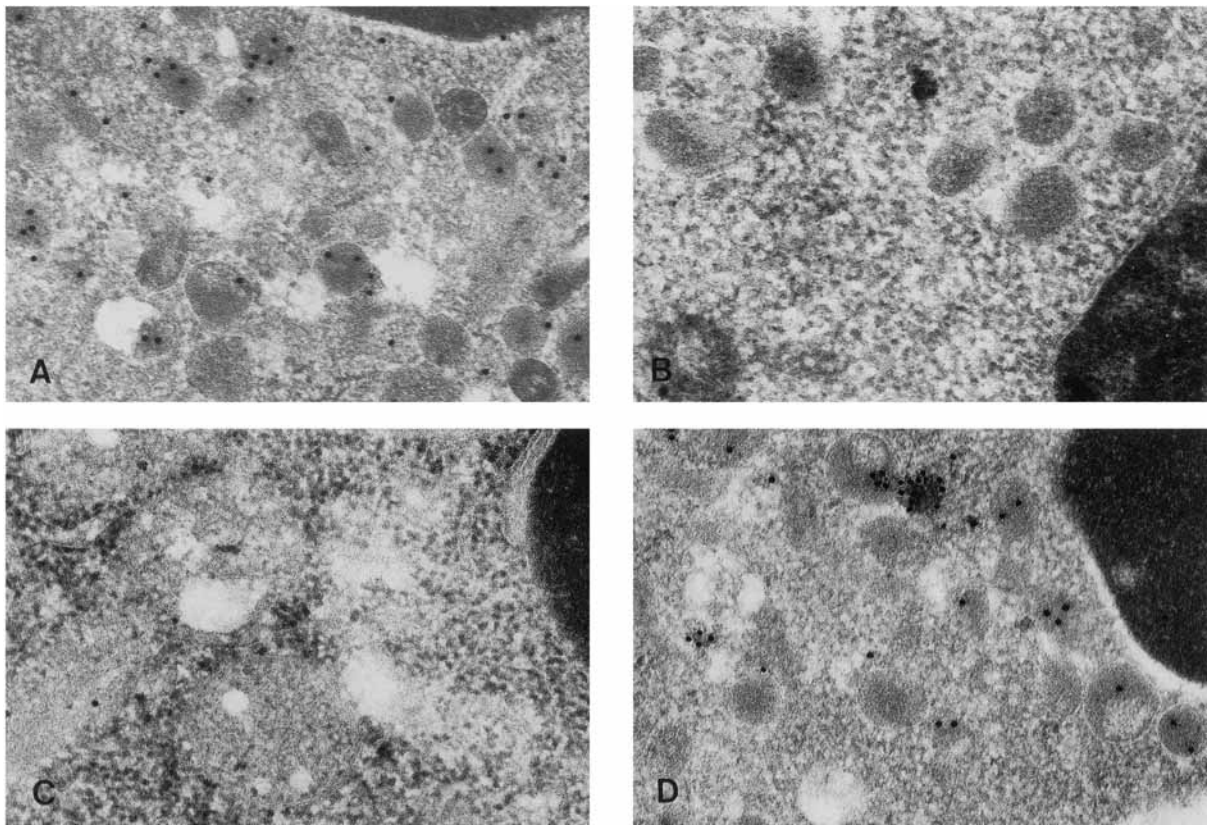


Fig. 7. Immunogold electron microscopy identifies B9 within a population of pleomorphic granules limited to neutrophils and their precursors (**A**). Incubation with preimmune serum demonstrates minimal background (**B**). When cultures of murine marrow treated for 3 days with GM-CSF were similarly studied, no staining for B9 could be detected either within the large pale primary granules or within the cytoplasm or other organelles (**C**). Anti-lysozyme (**D**) produced an identical pattern of staining to anti-B9. Original magnification, ×34,000.

ize the chromosomal structure of many of these genes. Those which are regulated during myeloid differentiation can be divided into several distinct groups, broadly classified as coding for ubiquitous ("housekeeping") proteins and myeloid specific proteins [Lubbert et al., 1991]. Many ubiquitously expressed proteins are necessary for cellular proliferation and survival. Myeloid specific proteins include those necessary for normal cellular differentiation, cellular recognition and cell-cell interactions, and the definition of a functional mature cell type. Cell lines established from human or murine leukemic blasts have frequently served as a homogeneous and well-defined population for production of cDNA libraries. These have been screened using a variety of strategies to produce a series of cloned and sequenced cDNAs corresponding to myeloid specific genes. The cell lines from which these genes have been isolated can be induced to differentiate along one or several pathways to become mature cells. However, several features of leukemic cell lines limit their comparability with normal non-leukemic counterparts. They frequently possess cytogenetic abnormalities, overexpress oncogenes, or show autocrine growth regulation. Consequently, the study of gene regulation in these cell lines may be problematic.

In order to circumvent the difficulties inherent to the use of transformed cell lines, we have developed a system to enable the study of gene expression during the process of differentiation in non-neoplastic bone marrow [Jaffe et al., 1988]. This model involves density gradient semi-purification of murine bone marrow and subsequent stimulation with recombinant GM-CSF. Equilibrium density gradient centrifugation permits an 11-fold enrichment of immature GM-CSF responsive cells. After 16–40 h in culture, GM-CSF non-responders (post-mitotic and mature cells) die and are replaced by a wave of GM-CSF responding cells. These responding cells are capable of proliferating and differentiating along granulocytic and monocytic lines. Because

the starting population is heterogeneous, the conditions of this system do not permit analysis of the immediate events post GM-CSF stimulation (days 0–1). However, there is a synchronous wave of proliferation and differentiation, such that by day 3 of culture an enriched population of promyelocytes is obtained.

Using this murine model system, a series of myeloid specific and differentiation stage specific clones were isolated by differential screening of a cDNA library. One clone, B9, was shown to represent a novel sequence with a pattern of mRNA accumulation similar to that noted for the early myeloid gene myeloperoxidase [Jaffe et al., 1988]. In this report, we present data to demonstrate that B9 is a component of neutrophilic granules, and that it is identified specifically in neutrophils and their precursors. Initial screening of both Northern and Southern blots prepared from human sources showed no cross-hybridization, even at low stringency. However, whole plasmid was used for these experiments, and the lack of cross hybridization may be secondary to steric inhibition of binding. Evaluation of the homology between B9 and porcine cathelin suggest limited sequence similarity confined to a conserved central core. Studies are in progress to evaluate the possibility of a human homologue with this conserved region.

Structural analysis of B9 suggests a relationship to members of a growing class of cysteine proteinase inhibitors (CPIs). Two distinct classes of CPIs are present in mammalian species: low molecular weight CPIs (stefin and cystatin) and high molecular weight CPIs (kininogen). These three distinct families form a superfamily, termed the cystatin superfamily, from which all members appear to have evolved from a common ancestral gene. The peptapeptide Gln-Val-Val-Ala-Gly is present in most known mammalian CPIs, and is believed to form part of the active site of the inhibitors [Barrett et al., 1986]. Following a meeting of the First International Symposium on Cysteine Proteinases and their inhibitors (Portoroz, Yugoslavia, September 15–18, 1985), it was agreed that the proteins that can be shown statistically to have an evolutionary relationship to chicken cystatin form the distinct cystatin superfamily. Family 1, the stefin family, contains stefin, cystatin A, and cystatin B. These proteins are distinguished by the lack of disulfide bonds and by close sequence homology to each other. Family 2, the cystatin family, contains proteins that have two disulfide bonds. These include chicken cystatin, human cystatin C, and human cystatin S. Family 3 contains the kininogens [Barrett et al., 1986].

Recently, members of two new families of cystatin-like proteins have been isolated and tentatively linked to the cystatin superfamily by sequence homology. One of these families contains a new cysteine proteinase inhibitor, cathelin, recently isolated from porcine leukocytes. Cathelin is a potent, tight-binding inhibitor of cathepsin L and papain, with no appreciable inhibition of other cysteine proteinases [Ritonja et al., 1989]. It contains 4 cysteine residues, which is characteristic of all cystatins. However, unlike all other cystatins, it does not contain methionine. Hence, it was proposed that this protein represented a new member of a new family of CPIs. The most highly conserved residue Gly9, found in all known sequences of inhibitory cystatins, recently identified as part of the active site of cystatin, is not present in cathelin. The N-terminus is 17 residues shorter than chicken cystatin. In addition, the highly conserved sequence "QXVXG" (residues 53–57) is not present either, suggesting that the interaction of cathelin with cysteine proteinases is not traditional [Kopitar et al., 1989].

We have isolated a novel cDNA from murine bone marrow which corresponds to a constituent of neutrophilic granules. Amino acid structural analysis identifies an amino terminal leader sequence which is most likely cleaved during post translational modification. This finding is common in secreted proteins, and is consistent with the immunolocalization of B9 protein. Few myeloid granule proteins have been cloned and sequenced, and little is currently known about the factors which regulate their expression. Previously analyzed myeloid specific genes like myeloperoxidase and lactoferrin are transcribed at a stage coincident with their packaging into granules [Jaffe et al., 1988], suggesting a mechanism for their specific localization. The fact that B9 is transcribed only in early myeloid cells (and not detected in segmented neutrophils) while it is packaged into secondary granules, suggests a novel mechanism of granule protein regulation. The possible presence of B9 in primary granules needs to be further investigated, as do alternative post-translational mechanisms of control (protein transport and processing). Unfortunately, in these studies, glutaraldehyde fixation necessary for optimum electron microscopic visualization decreased the staining intensity of

the B9 antibody, thus limiting our ability to resolve B9 protein within the Golgi, primary granules, and other cellular organelles. Limited structural homology to porcine cathelin, a member of the cystatin family of cysteine proteinase inhibitors, is apparent. Like cystatin, B9 lacks methionine and shows conservation of the 4 conserved cysteines. However, important structural differences exist, suggesting that B9 may in fact be a member of a new family. To our knowledge, this represents the first structural and cDNA sequence characterization of a neutrophil granule protein with homology to the cystatin superfamily. However, further studies are necessary to confirm the function of B9.

## ACKNOWLEDGMENT

This work was supported in part by Moffitt Cancer Center Research Grant Program 30018.

## REFERENCES

Arnaout MA, Remold-O'Donnell E, Pierce MW, Harris P, Tenen G (1988): Molecular cloning of the alpha subunit of human and guinea pig leukocyte adhesion glycoprotein Mo1: Chromosomal localization and homology to the alpha subunit of integrins. Proc Natl Acad Sci USA 85:2776.

Asamer H, Schmalzi F, Braunsteiner H (1971): Immunocytological demonstration of lysozyme (muramidase) in human leukemic cells. Br J Haematol 20:571–574.

Barker KA, Orkin SH, Newburger PE (1988): Expression of the X-CGD gene during induced differentiation of myeloid leukemia cell line HL-60. Mol Cell Biol 8:2804.

Barrett A, Fritz H, Grubb A (1986): Nomenclature and classification of the proteins homologous with the cysteine proteinase inhibitor chicken cystatin. Biochem J 236:312.

Bendayan M, Nanci A, Herbener GH (1986): A review of the study of protein secretion applying the protein A-gold immunocytochemical approach. Am J Anat 175:379–400.

Benson D, Boguski MS, Lipman DJ, Ostell J (1990): The National Center for Biotechnology Information. Genomics 6:389.

Bories D, Raynal MC, Solomon DH, Darzynkiewicz Z, Cayre YE (1989): Down-regulation of a serine protease, myeloblastin, causes growth arrest and differentiation of promyelocytic leukemia cells. Cell 59:959.

Castanon MJ, Spevak W, Adolf GR, Chlebowicz-Sledziewska E, Sledziewska A (1988): Cloning of human lysozyme gene and expression in the yeast *Saccharomyces cerevisiae*. Gene 66:223.

Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ (1979): Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18: 5294–5299.

Chung LP, Keshav S, Gordon S (1988): Cloning of the human lysozyme cDNA: Inverted Alu repeat in the mRNA and in situ hybridization for macrophages and Paneth cells. Proc Natl Acad Sci USA 85:6227.

Cronkite EP (1988): Analytical review of structure and regulation of hemopoiesis. Blood Cells 14:313–28.

Devereux JR, Haeberli P, Smithies O (1984): A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res 12:387.

Ferrero E, Goyert SM (1988): Nucleotide sequence of the gene encoding the monocyte differentiation antigen CD14. Nucleic Acids Res 16:4173.

Fouret P, DuBois RM, Bernaudin JF, Takahashi H, Ferrans VJ, Crystal RG (1989): Expression of the neutrophil elastase gene during human bone marrow cell differentiation. J Exp Med 169:833.

Goyert SM, Ferrero E, Rettig WJ, Yenamandra AK, Obata F, LeBeau MM (1988): The CD14 monocyte differentiation antigen maps to a region encoding growth factors and receptors. Science 239:497.

Gunning P, Ponte P, Okayama H, Engel J, Blau H, Kedes L (1983): Isolation and characterization of full-length cDNA clones for human α-, β-, and γ-actin mRNAs: Skeletal but not cytoplasmic actins have an amino-terminal cysteine that is subsequently removed. Mol Cell Biol 3:787–795.

Hickstein DD, Hickey MJ, Ozols J, Baker DM, Back AL, Roth GJ (1989): cDNA sequence for the alpha M subunit of the human neutrophil adherence receptor indicates homology to integrin alpha subunits. Proc Natl Acad Sci USA 86:257.

Jaffe BD, Sabath DE, Johnson GD, Moscinski LC, Johnson KR, Rovera G, Nauseef WM, Prystowsky MB (1988): Myeloperoxidase and oncogene expression in GM-CSF induced bone marrow differentiation. Oncogene 2:167–174.

Jameson BA, Wolf H (1988): The antigenic index: A novel algorithm for predicting antigenic determinants. Comput Appl Biosci 4:181.

Johnston J, Bollekens J, Allen RH, Berliner N (1989): Structure of the cDNA encoding transcobalamin I, a neutrophil granule protein. J Biol Chem 264:15754.

Kopitar M, Ritonja A, Popovic T (1989): A new type of low-molecular mass cysteine proteinase inhibitor from pig leukocytes. Biol Chem Hoppe Seyler 370:1145.

Leto TL, Lomax KJ, Volpp BD, Nunoi H, Sechler JMG, Nauseef WM, Clark RA, Gallin JI, Malech HL (1990): Cloning of a 67-kD neutrophil oxidase factor with similarity to a noncatalytic region of p60 (c-src). Science 248:727.

Look AT, Ashmun RA, Shapiro LH, Peiper SC (1989): Human myeloid plasma membrane glycoprotein CD13 (gp150) is identical to aminopeptidase N. J Clin Invest 83:1299.

Lubbert M, Herrmann F, Koeffler HP (1991): Expression and regulation of myeloid-specific genes in normal and leukemic myeloid cells. Blood 77:909–24.

Mason DY, Farrell C, Taylor CR (1975): The detection of intracellular antigens in human leukocytes by immunoperoxidase staining. Br J Haematol 31:361.

Moscinski LC, Prystowsky MB (1990): Identification of a series of differentiation-associated gene sequences from GM-CSF stimulated bone marrow. Oncogene 5:31–37.

Moscinski LC, Kasnic G, Saker A Jr (1992): The significance of an elevated serum lysozyme value in acute myelogenous leukemia with eosinophilia. Am J Clin Pathol 97:195–201.

Muller-Esterl W, Fritz H, Kellermann J (1985): Geneology of mammalian cysteine proteinase inhibitors. FEBS Lett 191:221.

Nagaoka I, Someya A, Iwabuchi K, Yamashita T (1992): Structure of the guinea pig neutrophil cationic peptide gene. FEBS Lett 303:31–35.

Parkos CA, Dinauer MC, Walker LE, Allen RA, Jesaitis AJ, Orkin SH (1988): Primary structure and unique expression of the 22-kilodalton light chain of human neutrophil cytochrome b. Proc Natl Acad Sci USA 85:3319.

Peiper SC, Ashmun RA, Look AT (1988): Molecular cloning, expression, and chromosomal localization of a human gene encoding the CD33 myeloid differentiation antigen. Blood 72:314.

Peltz GA, Grundy HO, Lebo RV, Yssel H, Barsh GS, Moore KW (1989): Human Fc gamma RIII: Cloning, expression, and identification of the chromosomal locus of two Fc receptors for IgG. Proc Natl Acad Sci USA 86:1013.

Ritonja A, Kopitar M, Jerala R, Turk V (1989): Primary structure of a new cysteine proteinase inhibitor from pig leucocytes. FEBS Lett 255:211.

Roth J (1986): Post-embedding cytochemistry with gold-labelled reagents: A review. J Microsc 143:125–137.

Royer-Pokora B, Kunkel LM, Monaco AP, Goff SC, Newburger PE, Baehner RL, Cole FS, Curnutte JT, Orkin SH (1986): Cloning the gene for an inherited human disorder-chronic granulomatous disease on the basis of its chromosomal location. Nature 322:32.

Sagoh T, Yamada M (1988): Transcriptional regulation of myeloperoxidase gene expression in myeloid leukemia HL-60 cells during differentiation into granulocytes and macrophages. Arch Biochem Biophys 262:599.

Salvesen G, Farley D, Shuman J, Przybyla A, Reilly C, Travis J (1987): Molecular cloning of human cathepsin G: Structural similarity to mast cell and cytotoxic T lymphocyte proteinases. Biochemistry 26:2289.

Sanger F, Nicklen S, Coulson AR (1977): DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463.

Simmons D, Seed B (1988): Isolation of a cDNA encoding CD33, a differentiation antigen of myeloid progenitor cells. J Immunol 141:2797.

Simmons DL, Tan S, Tenen DG, Nicholson-Weller A, Seed B (1989): Monocyte antigen CD14 is a phospholipid anchored membrane protein. Blood 73:284.

Takahashi H, Nukiwa T, Basset P, Crystal RG (1988): Myelomonocytic cell lineage expression of the neutrophil elastase gene. J Biol Chem 263:2543.

Tobler A, Miller CW, Johnson KR, Selsted ME, Rovera G, Koeffler HP (1988): Regulation of gene expression of myeloperoxidase during myeloid differentiation. J Cell Physiol 136:215.

Volpp BD, Nauseef WM, Donelson JE, Moser DR, Clark RA (1989): Cloning of the cDNA and functional expression of the 47-kilodalton cytosolic component of human neutrophil respiratory burst oxidase. Proc Natl Acad Sci USA 86:7195.

Wiedemann LM, Francis GE, Lamb RF, Burns JH, Winnie JN, MacKenzie ED, Birnie GD (1989): Differentiation stage-specific expression of a gene during granulopoiesis. Leukemia 3:227.