THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 268, No. 1, Issue of January 5, pp. 522-526, 1993
Printed in U.S.A.

The cDNA of the Neutrophil Antibiotic Bac5 Predicts a Pro-sequence Homologous to a Cysteine Proteinase Inhibitor That Is Common to Other Neutrophil Antibiotics*

(Received for publication, August 7, 1992)

Margherita Zanetti‡§, Giannino Del Sal‡¶, Paola Storici‡, Claudio Schneider¶, and Domenico Romeo‡

From the ‡Dipartimento di Biochimica, Biofisica e Chimica delle Macromolecole, Università di Trieste, I-34127 Trieste and the ¶Laboratorio Nazionale Consorzio Interuniversitario Biotecnologie, AREA Science Park, Padriciano, I-34012 Trieste, Italy

Bac5 is a 5-kDa proline- and arginine-rich antibiotic, stored as inactive precursor (proBac5) in the large granules of bovine neutrophils. A full-length cDNA encoding the precursor form of Bac5 has been cloned. The encoded protein (pre-proBac5) has a calculated mass of 20,031 Da and a pI of 9.21. This comprises a putative signal peptide of 29 amino acid residues and a 101-residue pro-sequence that precede the mature antibiotic. The pro-sequence is acidic and may neutralize the highly cationic Bac5, thus accounting for the inactivation of the antibiotic activity observed in *in vitro* experiments. The structure of mature Bac5 agrees closely with the amino acid sequence previously determined, with an additional tripeptide tail predicting carboxyl-terminal amidation. A valyl residue is deduced at the cleavage site for the proteolytic maturation of proBac5, consistent with a previous observation showing elastase as the enzyme involved in this processing step. The region upstream of Bac5 reveals high identity to corresponding regions of two neutrophil antimicrobial polypeptides, CAP18 from rabbit and bovine indolicidin. The COOH-terminal sequences of these antibiotics are completely unrelated. The pro-region also exhibits remarkable similarity to pig cathelin, an inhibitor of cathepsin L, indicating a common evolutionary origin.

Neutrophils are primary effector cells in the efferent limb of the immune response. They are rapidly attracted to infection sites, where they ingest noxious microbes into phagocytic vacuoles. Concomitantly, they release into the vacuoles highly reactive oxygen derivatives as well as a variety of antibiotic polypeptides (1-9). The effective concentrations and broad spectra of activity of these antibiotics, as tested in *in vitro* experiments, suggest that they may play a relevant role in host defense reactions. The neutrophil antibiotics of known sequence include the bactericidal/permeability-increasing protein, the defensins, and a family of proteins collectively named serprocidins, all contained in the azurophil granules (5, 10, 11).

Two polypeptides belonging to a new family of proline- and arginine-rich antibiotics, associated to the large granules of bovine neutrophils (12), have been purified and assayed for antimicrobial activity (13). The two polypeptides, named bactenecins or, in an abbreviated form, Bac5 and Bac7 (13), are synthesized in immature myeloid cells of the bone marrow and stored as inactive probactenecins in the large granules (12). Purified probactenecins do not display any antibiotic activity *in vitro* on organisms that are susceptible to the mature forms (14). Removal of the pro-portion, which results in appearance of the bactericidal activity, has been shown to be triggered by neutrophil stimulation with bacteria (8).

In this report we describe the cloning and sequence of a cDNA that encodes the precursor form of Bac5, and we compare the deduced amino acid sequence with that of other known proteins. The pro-portion of proBac5 has a high degree of homology to corresponding regions of two recently described neutrophil antimicrobial polypeptides, indolicidin (15, 16) and CAP18 (17). We also observe extensive identity of these pro-portions to cathelin, an inhibitor of the cysteine proteinase cathepsin L (18, 19), which may prevent proteolytic tissue damage at inflammatory sites. Thus, a common evolutionary origin may be shared by molecules that appear to exert complementary functions in host defense. The combination of antibiotic activity and tissue protection from inappropriate proteolysis may satisfy the requirements for an effective host response to both microbial infection and tissue injury.

EXPERIMENTAL PROCEDURES

RNA Preparation and Northern Analysis—Total RNA was extracted from bovine bone marrow cells (20).

Five μg of total RNA was separated by electrophoresis on 1% agarose gels containing 6.7% formaldehyde (21) and blotted onto nylon filters (GeneScreen Plus, Du Pont-New England Nuclear) using an LKB 2016 Vacu-Gene vacuum blotting system (Pharmacia LKB Biotechnology Inc.). RNA was cross-linked with UV-Stratalinker (Stratagene).

cDNA Library Construction and Screening—Poly(A$^+$) RNA was purified through oligo(dT)-cellulose chromatography (PolyA Quick mRNA purification kit, Stratagene). mRNA was quantified by using the DNA dipstick kit (Invitrogen). A random primed cDNA library was constructed using a cDNA synthesis kit (Pharmacia LKB Biotechnology Inc.). cDNA was size-selected on 1% agarose gel; the fraction up to 2 kb[1] was ligated to the λ T7/T3 vector (22) using *Eco*RI oligonucleotide adaptors. About 6 × 10^4 clones were screened

* This work was supported by grants from the National Research Council (Progetto Finalizzato Biotecnologie e Biostrumentazione) and from the Italian Ministry for University and Research. 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.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L02650.

§ To whom correspondence should be addressed: Dipartimento di Biochimica, Biofisica e Chimica delle Macromolecole, via A. Valerio, 38, I-34127 Trieste, Italy. Fax: 39-40-6763691.

[1] The abbreviations used are: kb, kilobase(s); nt, nucleotide(s); M-MLV, Moloney murine leukemia virus; bp, base pair(s).

by plaque hybridization with a degenerate oligonucleotide deduced from the Bac5 amino acid sequence. Filters were hybridized as described (23). Positive clones were isolated and cDNA fragments subcloned in Bluescript KS$^+$ (Stratagene).

Primer Extension Analysis and PCR Amplification of 3′ and 5′ Ends—For primer extension analysis, a 23-nt oligomer was annealed with 10 μg of total RNA. The extension was performed with Moloney murine leukemia virus (M-MLV) reverse transcriptase (BRL) for 1 h at 43 °C. Final products were analyzed on a 6% polyacrylamide sequencing gel.

PCR amplification of the 3′ end was performed by the rapid amplification of cDNA ends RACE protocol (24). Ten μg of total RNA was used to synthesize first strand cDNA with M-MLV reverse transcriptase. The reaction was performed in 20 μl using as a primer the oligonucleotide/adaptor 5′-TCGGATCCCTCGAGAAGC$(T)_{18}$-3′ at 40 °C with the buffer supplied. After 1 h, the mixture was heated to 95 °C and 80 μl of PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), containing 50 pmol of the upstream oligonucleotide 5′-CGCGAATTCAAAGCCTGTGAGCTTC-3′ (corresponding to nt 233–248 of the cDNA sequence) and of the downstream oligonucleotide/adaptor 5′-CGAGCTCGGATCCCTCGAGAAGCTT-3′, was added. Amplification was performed with 2.5 units of *Taq*I polymerase (Perkin Elmer-Cetus) for 30 cycles: 2 min at 94 °C, 1 min at 55 °C, and 3 min at 72 °C. To amplify the 5′ end, 10 μg of total RNA was used to synthesize the first strand using as a primer the oligonucleotide 5′-AGGCGGTAGAGATTAGCTTCTGA-3′, complementary to nt 162–184 of the cDNA sequence. Reverse transcription was performed with the M-MLV reverse transcriptase as above. The single-stranded cDNA obtained was tailed with terminal deoxynucleotidyl transferase (BRL) in a 30-μl reaction mixture using the supplied buffer containing 1 mM dGTP. Tailed cDNA was purified as described (25) and amplified as above, with an upstream primer complementary to the 20-dGTP tail and a downstream primer (5′-AGGAATTCGCTCATTGAACTGATCCAC-3′) located 4 nucleotides upstream of the 3′ end first strand primer. To assemble the full-length cDNA, 1 μg of total RNA was reverse-transcribed using the 3′ end oligonucleotide 5′-GAGAAGCTTCACAAGATTTTATTTTTTGG-3′, complementary to nt 637–658. The product was amplified with the same 3′ end primer and the 5′ end primer 5′-CAAGAATTCGGAGACTGGGGACCATGGAGA-3′ that corresponds to nt 1–21. Polymerase chain reaction conditions were as described above.

DNA Sequencing—cDNA was first sequenced directly in λ and then subcloned in pBluescript KS$^+$ vectors (Stratagene). The sequence was determined on double-stranded DNA (26) by the dideoxy chain termination method (27) using the T7 sequencing kit (Pharmacia LKB Biotechnology Inc.). Both strands were sequenced using universal and reverse primers and synthetic oligonucleotides deduced from the cDNA sequence. Regions with high G+C content were also sequenced in parallel with deazaguanosine (Pharmacia LKB Biotechnology Inc.) using an automated fluorescent DNA sequencer (EMBL Fluorescent DNA Sequencer). Compilation and analysis were performed using the IntelliGenetics Suite version 5.4 (IntelliGenetics Inc.)

Homology searches were carried out using the Swiss Prot data base.

The hydropathy plot was obtained according to Kyte and Doolittle (28).

Antibodies—The IgG fraction of rabbit antiserum to Bac5 was obtained as described (12).

In Vitro Transcription-Translation—The pBluescript KS$^+$ vector containing the full-length clone was linearized downstream with *Hin*dIII, and 1 μg of DNA was transcribed with T7 RNA polymerase in a 20-μl reaction volume containing 40 mM Tris-HCl, pH 8, 8 mM $MgCl_2$, 50 mM NaCl, 1 mM ATP, CTP, and UTP, 0.2 mM GTP, 1 mM cap analogue (Pharmacia LKB Biotechnology Inc.), 10 mM dithiothreitol, 35 units of RNasin (Pharmacia LKB Biotechnology Inc.), 20 units of T7 RNA polymerase (Stratagene) at 37 °C. Translation reactions were performed using rabbit reticulocyte lysate (Novagene) according to the manufacturer's instructions, with a final RNA concentration of 40 μg/ml. For membrane translocation analysis, the translation reaction was performed in the presence of 0.5 μl of undiluted dog pancreatic microsomes (Du Pont-New England Nuclear) for 1 h at 34 °C. Immunoprecipitation of translated products was performed as described. (29). Translation products were analyzed on a 15% SDS-polyacrylamide gel electrophoresis.

RESULTS

cDNA Cloning—A random hexamer-primed bovine bone marrow cDNA library was constructed in λ T7-T3/E-H vector. To develop a probe with which to screen the cDNA library, two 33-mer sets of antisense degenerate oligonucleotides were synthesized, based on the amino acid sequence of Bac5 determined by plasma desorption mass spectrometry (PD-MS) (30). The encoded sequence corresponds to residues 9–19 of mature Bac5 (amino acid residues 139–149 in Fig. 3). The two sets of oligonucleotides differ at a position corresponding to Arg (amino acid residue 142 in Fig. 3), resulting in a 16-fold degeneracy for *Ol*Bac5A and a 32-fold degeneracy for *Ol*Bac5B (Table I). By Northern analysis only *Ol*Bac5A was shown to recognize a 0.7-kb mRNA from bovine bone marrow (not shown) and was therefore used as a probe to screen the cDNA library by plaque hybridization. Five clones that gave duplicate signals after the primary screening were plaque-purified. The corresponding insert size was found to vary from 0.2 to 0.4 kb.

The clone containing the largest insert (393 bp, corresponding to nt 69–461 of the sequence in Fig. 3), incomplete at its 5′ and 3′ ends, was completely sequenced in both directions. This fragment encodes the first 19 amino acid residues of Bac5 present at its 3′ end. Northern analysis with the ^{32}P-labeled fragment reveals a transcript of about 0.7 kb in bovine bone marrow cells (Fig. 1*A*). No significant hybridization was observed in different bovine tissues, including heart, kidney, liver, lung, small intestine, spleen, and stomach (not shown).

The 5′ end of the mRNA was determined by primer extension analysis (Fig. 1*B*). Reverse transcription of bovine bone marrow cells mRNA from an oligonucleotide primer complementary to nt 162–184 shows that the start site of transcription occurs 184 nucleotides upstream of the primer sequence. This was later confirmed by the isolation and sequencing of the full-length cDNA, generated by the RACE strategy to the 3′ and the 5′ ends as in Fig. 2.

Features of the Predicted Structure—The complete cDNA sequence of 661 bp and its deduced amino acid sequence are shown in Fig. 3. A 531-nucleotide-long open reading frame starts at nucleotide 15. The predicted start codon is preceded by a short 5′-untranslated region of 14 nucleotides. The sequence flanking the putative translation start site at position 15 shows homology to the consensus sequence described by Kozak (31). A stop codon is found at position 543, followed by 116 bp of 3′-untranslated region. A typical polyadenylation signal is found at position 643, 19 nucleotides upstream of the poly(A) tail.

The mRNA encodes a protein of 176 residues (pre-proBac5) with a calculated mass of 20031 and an overall calculated pI of 9.21. The start methionine is followed by a 28-amino acid hydrophobic region (see the hydropathy plot in Fig. 4) corresponding to a putative signal peptide, as expected for a protein stored in cytoplasmic granules. The likely cleavage site for the signal peptidase is followed by a pro-portion including 11 positively and 19 negatively charged residues distributed over 101 residues, with a calculated pI of 4.14. Four Cys residues, clustered in the carboxyl-terminal moiety of the pro-sequence, indicate that there may be two intramolecular disulfide bonds in this region.

The cDNA sequence corresponding to mature Bac5 is at the 3′ end of the open reading frame (*underlined* in Fig. 3). Its deduced sequence matches the amino acid sequence previously determined (30), with the exception of a discrepant -Gly-Pro dipeptide predicted at positions 170–171 in place of a previously determined Arg residue. A -Gly-Arg-Arg tripeptide tail, not identified by amino acid analysis and protein

TABLE I

Degenerate oligonucleotide probes for Bac5

Both oligonucleotide sequences are antisense and are complementary to amino acid residues 139–149 of the deduced sequence. I*, inosine; R, A and G; Y, C and T.

Probe	Sequence 3′ → 5′											Degeneracy
	Pro	Pro	Ile	Arg	Pro	Pro	Phe	Tyr	Pro	Pro	Phe	
*Ol*Bac5A	GGI*	GGI*	TAR	GCI*	GGI*	GGI*	AAR	ATR	GGI*	GGI*	AAR	16
*Ol*Bac5B	GGI*	GGI*	TAR	YCT	GGI*	GGI*	AAR	ATR	GGI*	GGI*	AAR	32

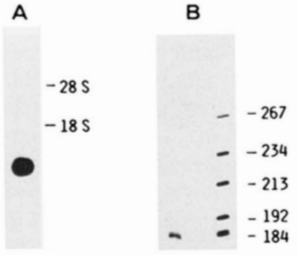

FIG. 1. **Northern analysis of total RNA from bovine bone marrow cells and transcription initiation site of Bac5 mRNA.** *A*, a Northern blot containing total RNA from bovine bone marrow cells was hybridized to the ^{32}P-labeled 393-bp fragment included between nt 69 and 461 of Bac5 cDNA. *B*, the start site for initiation of Bac5 mRNA was determined by primer extension analysis using a synthetic oligonucleotide complementary to nucleotides 162–184 of Bac5 cDNA. Total RNA from bovine bone marrow cells was hybridized to the primer and extended using reverse transcriptase. The extended product (*left*) was electrophoresed adjacent to size markers (*right*).

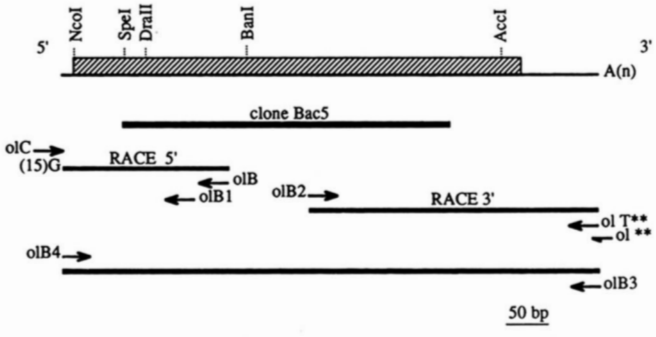

FIG. 2. **Partial restriction endonuclease map and sequencing strategy for cDNA encoding Bac5.** A schematic representation of the full-length cDNA of Bac5 is shown at *top*. Clone Bac5 was isolated by screening a bovine bone marrow cDNA library. *RACE 5′*: *(15)G* indicates a tail of dGTP; *olC*, 5′-GCGGAATTC(C)$_{15}$-3′; *olB*, 5′-AGGCGGTAGAGATTAGCTTCTGA-3′; *olB1*, 5′-AGGAATTCGCTCATTGAACTGATCCAC-3′. *RACE 3′*: *olT*** is the 5′-TCGGATCCCTCGAGAAGC(T)$_{18}$-3′ oligonucleotide/adaptor; *olB2*, 5′-CGCGAATTCAAAGCCTGTGAGCTTC-3′, *ol*** is the oligonucleotide/adaptor 5′-CGAGCTCGGATCCCTCGAGAAGCTT-3′. The full-length cDNA was generated using olB3 5′-GAGAAGCTTCACAAGATTTTATTTTTTGG-3′, and olB4 5′-CAAGAATTCGGAGACTGGGGACCATGGAGA-3′.

sequencing of Bac5, is also predicted at the carboxyl end.

In Vitro Transcription and Translation—To verify the deduced open reading frame encoding the gene of Bac5, the full-length cDNA cloned in Bluescript vector was transcribed and translated *in vitro*. The translation products were analyzed by immunoprecipitation with antibodies to purified Bac5.

The results indicate that the mRNA directs the synthesis of a polypeptide with an apparent M_r of 21000 (Fig. 5, *lane a*), not present in the control lanes (Fig. 5, *lanes b* and *c*). The apparent masses of the products obtained either in the presence (Fig. 5, *lane e*) or absence (Fig. 5, *lane d*) of microsomal membranes are in good agreement with previously reported values (12).

```
1                                              GGAGACTGGGGACC
15  ATGGAGACCCAGAGGGCCAGCCTCTCCCTGGGACGGTGTTCACTG
      M  E  T  Q  R  A  S  L  S  L  G  R  C  S  L  15
60  TGGCTACTGCTGCTGGGACTAGTGCTGCCCTCGGCCAGCGCCCAG
      W  L  L  L  L  G  L  V  L  P  S  A  S  A  Q  30
105 GCCCTCAGCTACAGGGAGGCCGTGCTTCGTGCCGTGGATCAGTTC
      A  L  S  Y  R  E  A  V  L  R  A  V  D  Q  F  45
150 AATGAGCGGTCCTCAGAAGCTAATCTCTACCGCCTCCTGGAGCTA
      N  E  R  S  S  E  A  N  L  Y  R  L  L  E  L  60
195 GACCCTACACCCAATGATGACTTGGACCCAGGCACCAGAAAGCCT
      D  P  T  P  N  D  D  L  D  P  G  T  R  K  P  75
240 GTGAGCTTCAGGGTGAAGGAGACCGATTGCCCCAGGACAAGCCAG
      V  S  F  R  V  K  E  T  D  C  P  R  T  S  Q   90
285 CAGCCCCTGGAGCAGTGTGACTTCAAGGAGAATGGGCTGGTGAAA
      Q  P  L  E  Q  C  D  F  K  E  N  G  L  V  K  105
330 CAGTGTGTGGGGACAGTCACCCTGGACCCATCAAATGACCAATTT
      Q  C  V  G  T  V  T  L  D  P  S  N  D  Q  F  120
375 GACATAAACTGTAATGAGCTTCAGAGTGTCAGATTTCGTCCACCA
      D  I  N  C  N  E  L  Q  S  V  R  F  R  P  P  135
420 ATCCGTCGTCCACCAATCCGTCCGCCGTTCTATCCACCGTTCCGC
      I  R  R  P  P  I  R  P  P  F  Y  P  P  F  R  150
465 CCGCCGATCCGCCCACCGATCTTCCCACCGATCCGGCCACCGTTC
      P  P  I  R  P  P  I  F  P  P  I  R  P  P  F  165
510 CGTCCACCCTTAGGACCGTTTCCTGGTAGACGGTGAACAATAGGC
      R  P  P  L  G  P  F  P  G  R  R  *              176
555 AGATAACTCCCTGATAAGGGCTTCTGATGAATCAGAAGCCCAGGG
600 AAGACCTCTTGGGATCTCTTTTGCCCTGAGTCAGCATCCAAAAAA
645 TAAAATCTTGTGAAAAC(A)n
```

FIG. 3. **Nucleotide and predicted amino acid sequence of Bac5.** The nucleotide sequence is *numbered* on the *left*. The first methionine is *numbered* on the *right* as amino acid 1. The *arrow* indicates the end of the putative signal sequence, including the first 29 amino acid residues. The sequence of the mature Bac5 is *underlined*. The amidation signal is indicated by a *double underline*, the stop codon is marked with an *asterisk*, and the polyadenylation signal is *overlined*. Cysteine residues are *boldface type*.

Sequence Comparisons—Homology searches through the Swiss-Prot Data Bank identified two known proteins that share 71 and 52% identity, respectively, with pre-proBac5: a specific inhibitor of cathepsin L, isolated from pig leukocytes and named cathelin (18, 19), and a lipopolysaccharide-binding protein from rabbit granulocytes, named CAP18 (17). The homology is confined to the region upstream of mature Bac5 (Fig. 6), and includes four invariant cysteine residues. Remarkable similarity (77% identity) is also exhibited by the same region of the recently sequenced indolicidin (16), another member of the repertoire of bactericidal peptides of bovine neutrophils (15).

DISCUSSION

Bac5 belongs to a proline- and arginine-rich family of antimicrobial peptides (13, 32). This polypeptide is stored in the large granules of bovine neutrophils as inactive proform or proBac5 (12). The mature Bac5 is generated by proteolytic cleavage catalyzed by elastase (14). Additional information is provided here on the structure and function of proBac5

through the sequencing of a cDNA representing Bac5 mRNA from bovine bone marrow cells.

cDNA analysis also allowed a better definition of the structure of mature Bac5, which was previously sequenced by PD-MS (30). A discrepant Arg assigned by PD mass spectra (30) is replaced in the cDNA sequence by a -Gly-Pro dipeptide predicted at positions 170–171 (Fig. 3). This discrepancy may arise from ambiguities in amino acid sequencing of the COOH-terminal moiety of Bac5. The mass of the -Gly-Pro dipeptide (154.17 Da) is very close to that of the Arg residue (156.19 Da), and the accuracy of mass determination by plasma desorption mass spectrometry might have not been high enough to appreciate this difference. The replacement of one Arg residue with -Gly-Pro is also consistent with the amino acid analysis of Bac5 (13).

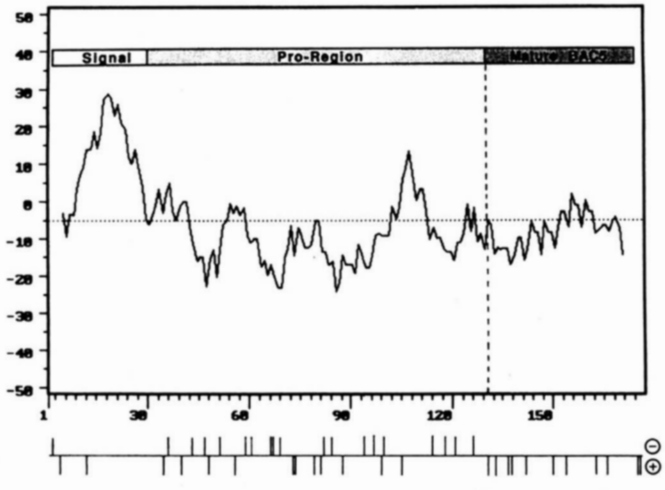

FIG. 4. **Hydropathy plot of Bac5.** Linear diagram of the sequence presented in Fig. 3, with the amino terminus on the *left* and the carboxyl terminus on the *right*. *Dashed vertical line*, cleavage position that produces the antimicrobial peptide. The *lower panel* indicates the distribution of basic (+) and acidic (−) residues. The hydropathy plot was produced by the method of Kyte and Doolittle (28) with a width of 11 residues.

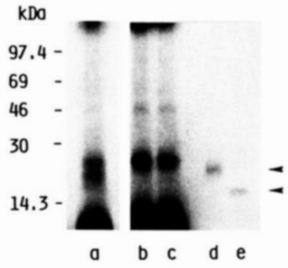

FIG. 5. ***In vitro* translation of mRNA transcribed from Bac5 cDNA.** The sense or the antisense sequences of Bac5 transcript were used to direct the *in vitro* protein synthesis in a reticulocyte lysate system in the presence of [^{35}S]methionine, and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Proteins synthesized in the presence of the sense (*lane a*) or the antisense (*lane b*) sequence of Bac5 transcript, and (*lane c*) in the absence of Bac5 transcript. *Lanes d* and *e* show the translation products (*arrows*) of the sense sequence of Bac5 transcript obtained in the absence and presence, respectively, of a microsomal membrane fraction, and immunoprecipitated with antibodies to Bac5.

The analysis of the cDNA sequence has given a relevant contribution to the elucidation of the structure and function of the pro-region. This sequence presents a putative valyl residue at the cleavage site for the proteolytic maturation of proBac5. Valine is one of the amino acids of choice for elastase, that has been shown to be the enzyme responsible for removal of the pro-portion (14). The pro-region is characterized by an alternate distribution of basic and acidic residues (Fig. 4), with a prevalence of acidic residues accounting for a calculated pI of 4.14. This structural characteristic may be critical for inactivation of the antibiotic activity, with the acidic pro-portion playing a role in neutralizing the highly cationic mature Bac5 (pI 13.38). The same hypothesis has also been advanced for other cationic toxic proteins (36).

A more precise characterization of the function of the pro-region has been aided by identification of sequence similarity with known proteins. Extensive identity has been found to corresponding regions of bovine neutrophil proindolicidin and rabbit neutrophil CAP18. The three proteins are likely to derive from a common gene family, as mismatches in the respective pro-sequences seem to rule out their origin through alternative splicing of the messenger. Interestingly, a common feature is the antimicrobial activity exerted by the structurally unrelated COOH-terminal portions cleaved from their precursors (14, 15, 17). Homologous pro-sequences present in

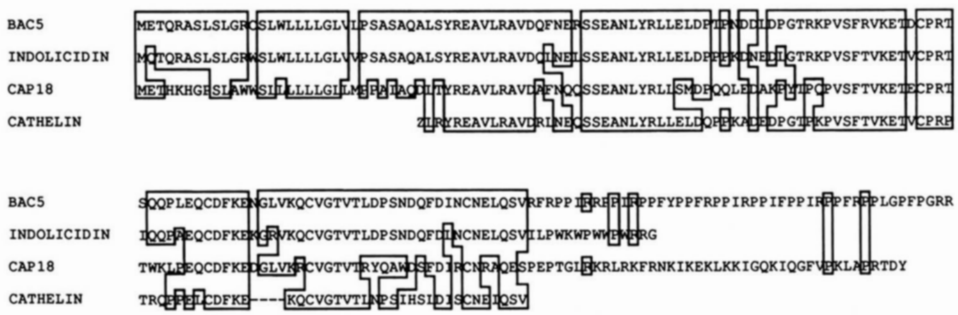

FIG. 6. **Alignment of Bac5 with indolicidin, cathelin, and CAP18.** The amino acid sequence deduced from cDNA encoding Bac5 is aligned with the sequence of bovine indolicidin (16) and rabbit CAP18 (17), both deduced from the cDNA, and with the sequence of pig cathelin determined by Edman degradation (18). *Boxed residues* reflect those common to proBac5 and at least one other family member. Sequences were aligned using the GENALIGN program.

different mammalian species may thus represent a common carrier for these, and perhaps other, defense peptides. However, this may be a hallmark of only a limited number of peptides, since other neutrophil antimicrobial proteins exhibit totally unrelated pro-sequences (11, 37–39).

Indirect evidence that the pro-sequence itself may play an active role independent of the antibiotic activity is given by the extensive identity shared with cathelin, a specific inhibitor of the cysteine proteinase cathepsin L isolated from pig leukocytes (19). Cathelin, which markedly differs from other families of cysteine proteinase inhibitors, appears to be a homologous protein (18) truncated at a position corresponding to the cleavage site of proBac5. Reasonably, sequences that are divergent copies of cathelin may also have retained the same function. Consistent with this hypothesis, purified proBac5 has proved to inhibit the *in vitro* activity of human cathepsin L, with a K_i of about 10^{-8} M.[2]

The fast extracellular release of proBac5 observed upon neutrophil stimulation with a soluble stimulus (8) may further support its contribution in limiting the elastinolytic activity of cathepsin L (40), with positive effects for the integrity of inflamed tissues. Although a similar inhibitory function has not been reported for proindolicidin and CAP18, we are tempted to speculate that all these pro-sequences are members of the same family of cysteine proteinase inhibitors. Their association with COOH-terminal sequences carrying antibiotic activity might have occurred through convergent evolution, as additional specificities, to serve new physiological functions.

Acknowledgments—We thank Licio Collavin for assistance in computer analysis and Francisco Baralle for critically reading the manuscript.

REFERENCES

1. Klebanoff, S. J. (1988) in *Inflammation: Basic Principles and Clinical Correlates* (Gallin, J. I., Goldstein, I. M., and Snydermann, R., eds) pp. 391–444, Raven Press, New York
2. Lehrer, R. I., Ganz, T., Selsted, M. E., Babior, B. M., and Curnutte, J. T. (1988) *Ann. Intern. Med.* **109**, 127
3. Joiner, K. A., Ganz, T., Albert, J., and Rotrosen, D. (1989) *J. Cell Biol.* **109** 2771–2782
4. Elsbach, P. (1990) *Trends Biotechnol.* **8**, 26–30
5. Lehrer, R. I., and Ganz, T. (1990) *Blood* **76**, 2169–2181
6. Spitznagel, J. K. (1990) *J. Clin. Invest.* **86**, 1381–1386
7. Gennaro, R., Romeo, D., Skerlavaj, B., and Zanetti, M. (1991) in *Blood Cell Biochemistry* (Harris, J. R., ed) pp. 335–368, Plenum Publishing Co., New York
8. Zanetti, M., Litteri, L., Griffiths, G., Gennaro, R., and Romeo, D. (1991) *J. Immunol.* **146**, 4295–4300
9. Zasloff, M. (1992) *Curr. Opin. Immunol.* **4**, 3–7
10. Elsbach, P., and Weiss, J. (1988) in *Inflammation: Basic Principles and Clinical Correlates* (Gallin, J. I., Goldstein, I. M., and Snydermann, R., eds) pp. 445–470, Raven Press, New York
11. Almeida, R., P., Melchior, M., Campanelli, D., Nathan, C., and Gabay, J. (1991) *Biochem. Biophys. Res. Commun.* **177**, 688–695
12. Zanetti, M., Litteri, L., Gennaro, R., Horstmann, H., and Romeo, D. (1990) *J. Cell Biol.* **111**, 1363–1371
13. Gennaro, R., Skerlavaj, B., and Romeo, D. (1989) *Infect. Immun.* **57**, 3142–3146
14. Scocchi, M., Skerlavaj, B., Romeo, D., and Gennaro, R. (1992) *Eur. J. Biochem.*, in press
15. Selsted, M. E., Novotny, M. J., Morris, W. L., Tang, Y.-Q., Smith, W., and Cullor, J. S. (1992) *J. Biol. Chem.* **267**, 4292–4295
16. Del Sal, G., Storici, P., Schneider, C., Romeo, D., and Zanetti, M. (1992) *Biochem. Biophys. Res. Commun.* **187**, 467–472
17. Larrick, J. W., Morgan, J. G., Palings, I., Hirata, M., and Yen, M. H. (1991) *Biochem. Biophys. Res. Commun.* **179**, 170–175
18. Ritonja, A., Kopitar, M., Jerala, R., and Turk, V. (1989) *FEBS Lett.* **255**, 211–214
19. Kopitar, M., Ritonja, A., Popovic, T., Gabrijelcic, D., Krizaj, I., and Turk, V. (1989) *Biol. Chem. Hoppe-Seyler* **370**, 1145–1151
20. Chomczinski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
21. Lehrach, H., Diamond, D., Wozney, J. M., and Boedtker, H. (1977) *Biochemistry* **16**, 4743–4750
22. Grimaldi, G., Manfioletti, G., and Schneider, C. (1987) *Nucleic Acids Res.* **15**, 9608
23. Church, G. M., and Gilbert, W. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 1991–1995
24. Frohman, A. M., Dush, K. M., and Martin, G. R. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 8998–9002
25. Del Sal, G., Ruaro, E., Philipson, L., and Schneider, C. (1992) *Cell* **70**, 595–607
26. Del Sal, G., Manfioletti, G., and Schneider, C. (1989) *BioTechniques* **7**, 514–520
27. Sanger, F., Nicklen, S., and Coulson, A., R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5463–5467
28. Kyte, J., and Doolittle, R. F. (1982) *J. Mol. Biol.* **157**, 105–132
29. Dasso, M. C., and Jackson, R. J. (1989) *Nucleic Acids Res.* **17**, 3129–3144
30. Frank, R. W., Gennaro, R., Schneider, K., Przybylski, M., and Romeo, D. (1990) *J. Biol. Chem.* **265** 18871–18874
31 Kozak, M. (1986) *Cell* **44**, 283–292
32. Agerberth, B., Lee, J., Bergman, T., Carlquist, M., Boman, H., G., Mutt, V., and Jornvall, H. (1991) *Eur. J. Biochem.* **202**, 849–854
33. Mains, R. E., Eipper, B. A., Glembotski, C. C., and Dores, R. M. (1983) *Trends Neurosci.* **6**, 229–235
34. Boman, H. G., Boman, I. A., Andreu, D., Li, Z.-q., Merrifield, R. B., Schlenstedt, G., and Zimmermann, R. (1989) *J. Biol. Chem.* **264**, 5852–5860
35. Gibson, B. W., Tang, D., Mandrell, R., Kelly, M., and Spindel, E. R. (1991) *J. Biol. Chem.* **266**, 23103–23111
36. Barker, R. L., Gleich, G. J., and Pease, L. R. (1988) *J. Exp. Med.* **168**, 1493–1498
37. Gray, P. W., Flaggs, G., Leong, S. R., Gumina, R. J., Weiss, J., Ooi, C. E., and Elsbach, P. (1989) *J. Biol. Chem.* **264**, 9505–9509
38. Daher, K., Lehrer, R. I., Ganz, T., and Kronenberg, M. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 7327–7331
39. Ganz, T., Rayner, J. R., Valore, E. V., Tumolo, A., Talmadge, K., and Fuller, F. (1989) *J. Immunol.* **143**, 1358–1365
40. Mason, R. W., Johnson, D. A., Barrett, A. J., and Chapman, H. A. (1986) *Biochem. J.* **233**, 925–927

[2] D. Verbanac and M. Zanetti, manuscript in preparation.