Developmental Regulation of Cryptdin, a Corticostatin/Defensin Precursor mRNA in Mouse Small Intestinal Crypt Epithelium

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Abstract. Cryptdin mRNA codes for the apparent precursor to a corticostatin/defensin-related peptide that accumulates to high levels in mouse intestinal crypt epithelium during postnatal development. The primary structure, intestinal cell distribution, and developmental appearance of cryptdin mRNA have been determined. Cryptdin mRNA is 450–480 nucleotides long. Translation of the partial cryptdin cDNA sequence reveals a 70–amino acid open reading frame that includes 32 carboxy-terminal residues that align with those in the consensus sequence, C.CR... C....ER..G.C...G....CCR, which is a common feature of leukocyte defensins and lung corticostatins (Selsted, M. E., D. M. Brown, R. J. DeLange, S. S. L. Harwig, and R. I. Lehrer. 1985. *J. Biol. Chem.* 260:4579–4584; Zhu, Q., J. Hu, S. Mulay, F. Esch, S. Shimasaki, and S. Solomon. 1988. *Proc. Natl.* *Acad. Sci. USA.* 85:592–596). *In situ* hybridization of cryptdin cDNA to paraformaldehyde-fixed, frozen sections of adult jejunum and ileum showed intense and specific labeling of epithelial cells in the base of all crypts. Analysis of sections from suckling mice showed that cryptdin mRNA is detectable in 10–20% of crypts in 10-d-old mice, in ~80% of crypts in 16-d-old mice, and in all crypts of mice 20 d and older. During the fourth week, the sequence accumulates in crypts to the maximal adult level. Cryptdin mRNA content in adult small intestine is independent both of T cell involvement and luminal bacteria. The role of cryptdin in small bowel physiology remains to be determined: cryptdin may inhibit bacterial translocation, modulate intestinal hormone synthesis, influence hormonal sensitivity of the intestinal epithelium, or exhibit a multiplicity of related activities.

CORTICOSTATINS (CS)[1] and defensins constitute a family of structurally related cysteine-rich peptides. Low molecular weight, cationic polypeptides termed "defensins" have been isolated from rabbit, human, and guinea pig neutrophils and from rabbit alveolar macrophages (Selsted et al., 1985a,b; Daher et al., 1988; Selsted and Harwig, 1987; Selsted et al., 1983). All defensins and CSs contain 31–33 amino acids, including a consensus sequence of 11–12 residues in which cysteine and arginine residues align in a highly conserved fashion (Fig. 1). Despite sharing a structural "core," defensin sequences diverge markedly between positions 5 and 11 and between residues 19 and 27 (Fig. 1, regions A and B, respectively).

Although the extent to which amino acid substitutions in domains A and B (Fig. 1) determine variability in microbicidal potency (Ganz et al., 1985b; Selsted et al., 1984) and corticostatic activity (Zhu et al., 1988, 1989) remains to be defined, molecules bearing the consensus motif appear to have varied, perhaps multiple, functions in situ. For example, two macrophage defensins, MCP-1 and MCP-2, have antiviral and microbicidal activities when assayed in vitro

(Lehrer et al., 1983). On the other hand, rabbit lung CS I, CS II, CS III, and CS IV, which are identical to rabbit defensins NP-3a, NP-3b, MCP-1 (NP-1), and MCP-2 (NP-2), respectively, inhibit ACTH-stimulated corticosteroid production to varying degrees in rat adrenal cells in culture (Zhu et al., 1988, 1989). Here, we describe cryptdin, a sequence that contains the CS/defensin consensus and is developmentally regulated in crypt epithelial cells that lie below the level of stem cells in the mouse small intestine.

The rodent small intestine continues to differentiate in neonates, particularly during the third and fourth weeks (Henning, 1985; Klein and McKenzie, 1983a,b; Raul et al., 1977). During this period, profound changes occur in the cell kinetics, transit time, epithelial enzyme content, transport properties, and macromolecular permeability of the columnar epithelium (Koldovsky et al., 1966; Moog, 1962; Herbst and Sunshine, 1969; Altman and Enesco, 1967). The effectors and mediators of these events are not well-understood. To study genetic regulation during the differentiation and maturation of the small bowel, we have been investigating abundant intestinal mRNAs that code for 6-kD, cysteine-rich polypeptides which accumulate in postnatal development (Ouellette and Cordell, 1988). Cryptdin mRNA, previously

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1. *Abbreviation used in this paper:* CS, corticostatin.

A   B

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CYS ___ CYS ARG ___ ___ ___ CYS ___ ___ ___ GLU ARG ___ ___ GLY ___ CYS ___ ___ ___ GLY ___ ___ ___ ___ ___ CYS CYS ARG

Figure 1. The corticostatin/defensin consensus sequence. Residues shown denote amino acids of the CS/defensin consensus sequence. Underscored spaces indicate positions where sequences of CS/defensins diverge.

termed asb4/134, is one member of this class, and we now report on its coding function, site of intestinal expression, and aspects of its developmental regulation.

Materials and Methods

Animals

Outbred Swiss mice [(Crl:CD-1)(ICR)BR] were 45-d-old males (30–35 g) or timed-pregnant dams from Charles River Breeding Laboratories, Inc. (Wilmington, MA); nude mice were Swiss-nu/nu purchased from Taconic Farms, Inc. (Germantown, NY); 28-d-old germ-free National Institutes of Health Swiss mice, shipped aseptically, also were purchased from Taconic Farms, Inc. Mice were housed under 12-h cycles of light and dark and had free access to standard rat chow and water.

Isolation of RNA from Mouse Small Intestine

RNAs were deproteinized by selective precipitation from guanidine isothiocyanate and guanidine hydrochloride (Chirgwin et al., 1979) followed by exhaustive extraction with phenol/chloroform/isoamyl alcohol (Perry et al., 1972; Ouellette and Cordell, 1988). After flushing with 50 ml ice-cold water, the small bowel was homogenized in 20 vol 4 M guanidine isothiocyanate at room temperature. Except for in situ hybridization, samples of small bowel included the region from the pylorus to the ileocecal valve.

Blot Hybridization

Hybridization experiments were conducted using DNA probes labeled with [^{32}P]dCTP by nick-translation (Rigby et al., 1977) or with Klenow fragment after random oligonucleotide priming (Feinberg and Vogelstein, 1983, 1985). For RNA transfer blots (Northern blots), RNAs were separated in morpholino propane sulfonic acid (MOPS) formaldehyde-containing 2% agarose gels (Maniatis et al., 1982; Rave et al., 1979) and transferred to Gene Screen Plus membranes (DuPont Co., Wilmington, DE; New England Nuclear, Boston, MA) by capillarity (Thomas, 1980). Blots were hybridized and washed according to the recommendations of the manufacturer.

In Situ Hybridization

Freshly dissected mouse organs were fixed for 18 h at 4°C by immersion in 10 vol phosphate-buffered 4% paraformaldehyde solution containing 0.05% diethylpyrocarbonate, soaked overnight at 4°C in 30% sucrose in PBS, frozen in O.C.T. compound (Miles-Yeda Inc., Elkhart, IN) in dry ice-isopentane, and stored at −70°C before sectioning. Sections (5 μm) on slides coated with 50 μg/ml poly-L-lysine (Huang et al., 1983) were hybridized 18 h to [^{35}S]dCTP-labeled DNA probes (20,000–300,000 cpm) in 20 μl buffer containing 0.3 M NaCl, 10 mM Tris (pH 7.5), 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% BSA, 1 mM EDTA, 10 mM DTT, 0.025% yeast tRNA, 10% dextran sulfate, and 50% deionized formamide (Angerer et al., 1987; Singer et al., 1986). Sections were sealed under coverslips using rubber cement, and slides were incubated overnight at 50°C in humidified polyethylene boxes. Slides were washed three times with gentle agitation, in 4× SSC at 37°C for 20 min per wash, twice in 2× SSC for 30 min at 37°C, and once with 0.1× SSC for 30 min at 37°C. After dehydration with successive ethanol washes in 0.3 M ammonium acetate, slides were coated with NTB-2 emulsion (Eastman Kodak Co., Rochester, NY), incubated at −20°C for 2–10 d, developed, and stained with hematoxylin and eosin.

DNA Sequence Analysis

Cryptdin cDNA was subcloned into m13mp8/9 vectors and both strands

were sequenced by dideoxynucleotide random termination (Sanger et al., 1977) using [^{35}S]dATP with modified Sequenase (United States Biochemical Corp., Cleveland, OH) or Sequel SS (International Biotechnologies, Inc., New Haven, CT) DNA sequencing reagents. Sequencing gels were run according to the method of Biggin et al. (1983). Sequences were assembled and analyzed using the programs of Staden (1984, 1986) and the University of Wisconsin Genetics Computer Group (Devereux et al., 1985). The National Biomedical Research Foundation Protein Information Resource protein sequence database (George et al., 1986) was searched for similarities to cryptdin and matches were aligned using IFIND (IntelliGenetics, Inc., Mountain View, CA) on the BIONET™ National Computer Resource for Molecular Biology (Kristofferson, 1987).
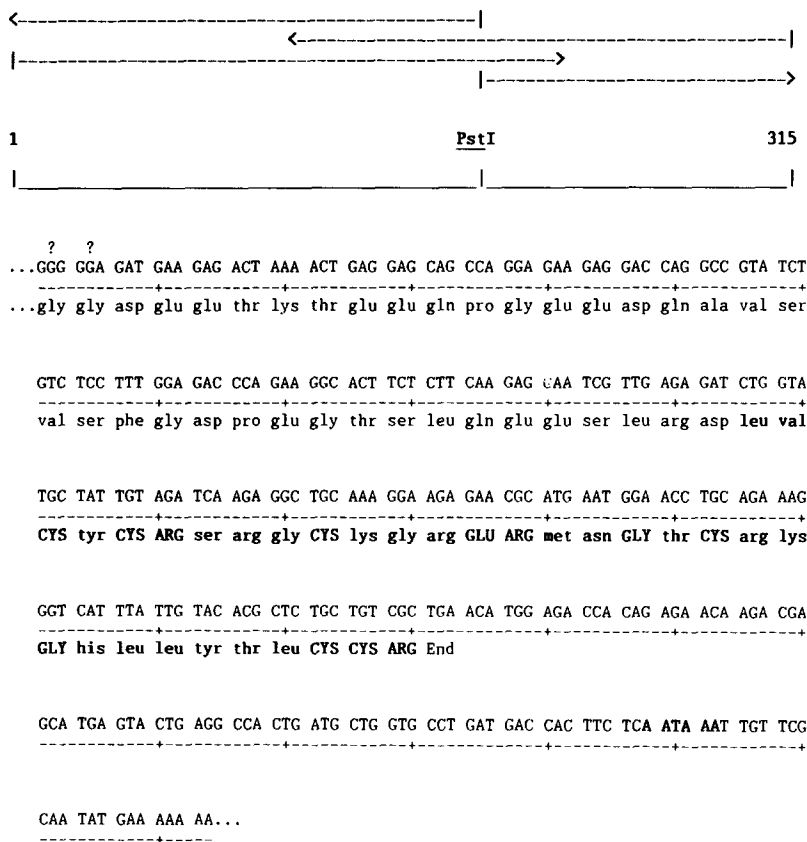
Results

Cryptdin cDNA corresponds to a developmentally regulated intestinal LMW mRNA. In a previous report, cryptdin (asb4/134) mRNA was shown to code for a 6-kD, cysteine-rich polypeptide, to accumulate in the third to fourth weeks of postnatal development, to be abundant in adult jejunum and ileum but not detectable in duodenum or colon, and to be present in testis and brain (Ouellette and Cordell, 1988).

Cryptdin cDNA Sequence Analysis

To help define the role of cryptidin, its sequence and coding function were determined (Fig. 2). The translated partial cDNA sequence contains a single 70–amino acid open reading frame in which the COOH-terminal half of the molecule contains all six cysteine residues. The translation product is a highly polar protein. The carboxy end is strongly cationic in that nine of the 30 COOH-terminal amino acids contain basic sidechains (Fig. 2 B). In contrast to this basic region, 12 of the 40 NH_2-terminal residues are acidic. In addition to structural stability provided by potential disulfide bonds, interactions between positively and negatively charged residues in this precursor may be important in its posttranslational modification (see below).

A comparison of the cryptdin open reading frame with protein sequences in the National Biomedical Research Foundation Protein Information Resource database (George et al., 1986) showed an unmistakable relation between cryptdin and CS/defensin cationic peptides. The COOH-terminal half of the cryptdin peptide sequence contains the CS/defensin consensus demonstrating its unambiguous structural similarity to this family of peptides (Fig. 3 A). Overall, residues 39–70 of cryptdin are 47% identical with peptides MCP-1 (CS III) and MCP-2 (CS IV) from rabbit macrophages and neutrophils (Fig. 3 A), and its similarity with MCP-2 increases to 70% if conservative amino acid substitutions are taken into account (not shown). Alignment of the deduced cryptdin polypeptide sequence with that of the complete human defensin precursor prepro-hnp-1 (Daher et al., 1988) showed identity at an additional nine residues in the amino terminal portion of the cryptdin molecule (Fig. 3 B). However, alignment of both cryptdin and prepro-hnp-1 protein sequences with full-length rabbit prepro-MCP-1 (Ganz, T., and F. Fuller,

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                         <-------------------------------------------------|
|--------------------------------------------------------->
                                 |------------------------------------>

1                                PstI                          315

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  ?   ?
...GGG GGA GAT GAA GAG ACT AAA ACT GAG GAG CAG CCA GGA GAA GAG GAC CAG GCC GTA TCT
   -----------+------------+------------+------------+------------+------------+
...gly gly asp glu glu thr lys thr glu glu gln pro gly glu glu asp gln ala val ser


GTC TCC TTT GGA GAC CCA GAA GGC ACT TCT CTT CAA GAG CAA TCG TTG AGA GAT CTG GTA
-----------+------------+------------+------------+------------+------------+
val ser phe gly asp pro glu gly thr ser leu gln glu glu ser leu arg asp leu val


TGC TAT TGT AGA TCA AGA GGC TGC AAA GGA AGA GAA CGC ATG AAT GGA ACC TGC AGA AAG
-----------+------------+------------+------------+------------+------------+
CYS tyr CYS ARG ser arg gly CYS lys gly arg GLU ARG met asn GLY thr CYS arg lys


GGT CAT TTA TTG TAC ACG CTC TGC TGT CGC TGA ACA TGG AGA CCA CAG AGA ACA AGA CGA
-----------+------------+------------+------------+------------+------------+
GLY his leu leu tyr thr leu CYS CYS ARG End


GCA TGA GTA CTG AGG CCA CTG ATG CTG GTG CCT GAT GAC CAC TTC TCA ATA AAT TGT TCG
-----------+------------+------------+------------+------------+------------+


CAA TAT GAA AAA AA...
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Figure 2. Analysis of cryptdin cDNA sequence. Cryptdin cDNA was sequenced by dideoxynucleotide random termination (see Materials and Methods) as illustrated in *A*. The sequence shown in *B* was assembled and translated (Materials and Methods) to identify the single open reading frame shown. Residues corresponding to the CS/defensin consensus are shown in uppercase, bold face type. The first two glycine residues are not known with certainty, because the cDNA library was constructed by homopolymeric tailing (Ouellette and Cordell, 1988).

personal communications) showed that only the asp-glu match at cryptdin residues 2-3 (HNP-1 residues 27-28) are conserved among these three sequences. As reported for HNP-1 (Daher et al., 1988), comparisons of the amino terminal portion of cryptdin with sequences in Protein Information Resource failed to identify any known related proteins (data not shown). RNA sequencing has shown that the 5' end of cryptdin mRNA extends an additional 115 nucleotides and codes, as in the case of HNP-1 (Daher et al., 1988) and MCP-1 (T. Ganz, personal communication) for a hydrophobic leader (data not shown). Although the cryptdin cDNA clone is not full-length, the existing data are unambiguous in demonstrating that cryptdin mRNA codes for the propeptide form of a CS/defensin-related precursor of which the 32 COOH-terminal amino acids constitute the final product. The fact that the cryptdin gene locus maps to the proximal region of chromosome 8 (Ouellette, A. J., D. Pravtcheva, F. H. Ruddle, and M. James, manuscript submitted for publication) at a homologous position to the HNP-1 locus in the human (R. S. Sparkes, personal communication) provides further evidence of the relation of cryptdin to the defensins.

The similarity of cryptdin to other CS/defensins, is lower than that to MCP-1 and MCP-2 (Fig. 3 *A*). However, these estimates of apparent identity may be misleading, since, for several CS/defensins, the identity with cryptdin is almost wholly restricted to consensus residues. If consideration is limited to amino acids outside the consensus sequence, cryptdin and MCP-1 (CS III) and MCP-2 (CS IV) are identical at only 2 of 19 and 3 of 19 positions, respectively (Fig. 3 *C*). Recently, several cryptdin-related cDNA clones have

been isolated whose DNA sequences are 90% identical with the 5' half of cryptdin mRNA but diverge in their 3' sequences (Lualdi, J. C., and A. J. Ouellette, unpublished data). Thus, other cryptdin-related mRNAs exist in the small bowel.

Because analysis of whole organ RNA may be complicated by the dynamics and diversity of cell populations in complex organs, intestinal sections were hybridized to cryptdin cDNA probes to identify the intestinal cell population in which cryptdin mRNA is most abundant.

Cryptdin mRNA in Adult Small Bowel Crypts

In situ hybridization experiments showed that cryptdin mRNA is abundant in the basal epithelium of adult small intestinal crypts (Fig. 4). Because of the similarity of cryptdin to CS/defensins from cells of myeloid origin, leukocytes in the lamina propria were expected to be the primary intestinal cell population containing cryptdin mRNA. Two cell populations in adult mouse jejunum and ileum reacted strongly with the cryptdin probe: epithelial cells at the base of each crypt, and, less consistently, apparent leukocytes within the villus lamina propria (Fig. 4, *A* and *B*, and Fig. 5, *C* and *D*). Because M13 (not shown) and pBR322 (Fig. 4 *C*) DNA control probes hybridized occasionally to lamina propria leukocytes, the occurrence of cryptdin or cryptdin-related mRNAs in these cells is still unclear. However, as shown in Fig. 5, *A-C*, lamina propria cells positive for cryptdin mRNA are not detectable in mice <20 d old, suggesting that hybridization to these cells is specific. The definitively positive cells in epithelial crypts occupy the zone below stem cell epithelium

Three-letter code alignment (A) — part 1

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Cryptdin	leu	val	**CYS**	tyr	**CYS**	**ARG**	ser	arg	gly	**CYS**	lys	gly	arg	**GLU**	**ARG**	met	asn
Consensus	—	—	**CYS**	—	**CYS**	**ARG**	—	—	—	**CYS**	—	—	—	**GLU**	**ARG**	—	—
MCP-1	val	val	CYS	ala	CYS	ARG	arg	ala	leu	CYS	leu	pro	arg	GLU	ARG	arg	ala
MCP-2	val	val	CYS	ala	CYS	ARG	arg	ala	leu	CYS	leu	pro	leu	GLU	ARG	arg	ala
NP-3a	gly	ile	CYS	ala	CYS	ARG	arg	arg	phe	CYS	pro	asn	ser	GLU	ARG	phe	ser
NP-3b	gly	arg	CYS	val	CYS	ARG	lys	gln	2leu	CYS	ser	tyr	arg	GLU	ARG	arg	ile
NP-4	val	ser	CYS	thr	CYS	ARG	arg	phe	ser	CYS	gly	phe	gly	GLU	ARG	ala	ser
NP-5	val	phe	CYS	thr	CYS	ARG	gly	phe	leu	CYS	gly	ser	gly	GLU	ARG	ala	ser
CSI	gly	ile	CYS	ala	CYS	ARG	arg	arg	phe	CYS	pro	asn	ser	GLU	ARG	phe	ser
HNP-1	ala/asp		CYS	tyr	CYS	ARG	ile	pro	ala	CYS	ile	ala	gly	GLU	ARG	arg	tyr
GPNP-1			CYS	ile	CYS	thr	thr	arg	thr	CYS	arg	phe	pro	tyr	tyr	ARG	leu

Three-letter code alignment — part 2

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Cryptdin	**GLY**	thr	**CYS**	arg	lys	**GLY**	his	leu	leu	tyr	thr	leu	**CYS**	**CYS**	**ARG**		
Consensus	**GLY**	—	**CYS**	—	—	—	**GLY**	—	—	—	—	—	**CYS**	**CYS**	**ARG**		
MCP-1	GLY	phe	CYS	arg	ile	arg	GLY	arg	ile	his	pro	leu	CYS	CYS	ARG	arg	
MCP-2	GLY	phe	CYS	arg	ile	arg	GLY	arg	ile	his	pro	leu	CYS	CYS	ARG	arg	
NP-3a	GLY	tyr	CYS	arg	val	asn	GLY	ala	arg	tyr	val	arg	CYS	CYS	ser	ARG	arg
NP-3b	GLY	asp	CYS	lys	ile	arg	GLY	val	arg	phe	pro	phe	CYS	CYS	pro	ARG	
NP-4	GLY	ser	CYS	thr	val	asn	GLY	val	arg	his	thr	leu	CYS	CYS	ARG	arg	
NP-5	GLY	ser	CYS	thr	ile	asn	GLY	val	arg	his	thr	leu	CYS	CYS	ARG		
CSI	GLY	tyr	CYS	arg	val	asn	GLY	ala	arg	tyr	val	arg	CYS	CYS	ser	ARG	ARG
HNP-1	GLY	thr	CYS	ile	tyr	gln	GLY	arg	leu	trp	ala	phe	CYS	CYS			
GPNP-1	GLY	thr	CYS	ile	phe	gln	asn	arg	val	tyr	thr	phe	CYS	CYS			

One-letter code alignment (B/C)

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CRYPTDIN   V C Y C R  S R G  C  K G R  E R  M N  G  T  C  R K  G  H L L Y T L  C C R
Consensus  C - C R - - -  C  - - -  E R  - -  G  -  C  - - -  G  - - - - -  C C R

MCP-1   V  .  .  .  .  .  .  .  .  R  .  .  .  .  .  .  R  .  .  .  .  .  .  .  L  .  .  .
MCP-2   V  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  R  .  .  .  .  .  .  .  L  .  .  .
NP-5    .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  L  .  .  .
NP-4    .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  L  .  .  .
NP-3A   .  .  .  .  .  R  .  .  .  .  .  .  .  .  .  .  R  .  .  .  .  .  .  Y  .  .  .  .
NP-3B   .  .  .  .  .  .  .  .  .  R  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
gpnp-1  .  .  .  .  .  R  .  .  .  .  .  .  .  T  .  .  .  .  .  .  .  .  Y  T  .  .  .
hnp-1 (A/D)  . Y  .  .  .  .  .  .  .  .  .  .  .  T  .  .  .  .  .  .  L  .  .  .  .  .  .
CSI     .  .  .  .  .  R  .  .  .  .  .  .  .  .  .  .  R  .  .  .  .  .  .  .  .  .  .
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                                                             1
Cryptdin   ...  ...  ...  ...  ...  ...  ...  ...  .GD EET KTE
                                                              |  |
HNP-1      MRT LAI LAA ILL VAL QAQ AEP LQA RAD EVA AAP

Cryptdin   EQP GEE DQA VSV SFG DPE GTS LQE ESL RD_ LV
           ||          | | |      |            |
HNP-1      EQI AAD IPE VVV SLA VDE SLA PKH PGS RKN MA

Cryptdin   CYC RSR GCK GRE RMN GTC RKG HLL YTL CCR
           ||| |      | |   |    | |    |||            ||
HNP-1      CYC RIP ACI AGE RRY GTC IYQ GRL VAF CC
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Figure 3. Alignment of cryptdin peptide sequence with defensins and corticostatins. (A) Amino acid residues 39–70 of the cryptdin peptide sequence (Fig. 2 B) are shown aligned with a full-length peptide sequences of human, rabbit, and guinea pig defensins and rabbit corticostatin I. Three-letter code showing alignments with cryptdin as determined by WORDSEARCH (Devereux, 1985). MCP-1 and MCP-2, rabbit alveolar macrophage and neutrophil defensins corresponding to CS III and CS IV; NP-3A, NP-3B, (CS I and CS II, respectively), NP-5, NP-4, rabbit peritoneal neutrophil defensins and corticostatins; GPNP-1, guinea pig peritoneal neutrophil defensin; HNP-1, human neutrophil defensin; CS I, fetal rabbit lung corticostatin I (NP-3A). Consensus residues are shown in bold, uppercase notation. (B) The cryptdin protein sequence is shown aligned in single letter notation with prepro-HNP-1 protein sequence (Daher et al., 1988). Residues of the mature peptides are denoted by bolded typeface. (C) One-letter amino acid notation showing alignment of only nonconsensus residues.

which includes Paneth cells and less abundant neuroendocrine cells.

Cryptdin cDNA hybridized to crypt epithelial cells with great specificity and reproducibility. Of several control and cDNA probes applied to intestinal sections, only cryptdin cDNA hybridized to cells in crypts. The reproducibility of these experiments (Fig. 4, A and B, and Fig. 5) is demonstrated by the fact that samples of small intestine from six individual mice analyzed in five separate hybridization experiments with varying quantities of five different preparations of probe gave identical hybridization results. Consistent with previously reported northern blot results (Ouellette and Cordell, 1988), in situ hybridization of cryptdin cDNA to both liver (Fig. 4 E), and colon (Fig. 4 F) sections was negative.

For possible insights into the intestinal role of cryptdin, sections of adult mouse testis were hybridized with cryptdin cDNA to identify the testis cell population that contains cryptdin mRNA (Fig. 4 D). Previously, Northern blots had shown that cryptdin mRNA is present in poly(A)$^+$ mRNA from testis and brain at <1% the level in the full-length, full-thickness small bowel (Ouellette and Cordell, 1988). Be-

cause cryptdin mRNA is abundant in crypt basal epithelium (Fig. 4, A and B) and CS I was isolated from fetal lung, cryptdin mRNA had been expected to occur in less well-differentiated testis cell populations. No cell population in testis hybridized strongly to the cryptdin probe, but spermatogonia and immature spermatocytes consistently had three- to fourfold higher grain counts than other cells in testis.

Developmental Appearance of Cryptdin mRNA

In the second week of postnatal development, individual crypts begin to accumulate cryptdin mRNA independently. To distinguish developmental induction of cryptdin mRNA in resident cells from introduction of the sequence to the gut by invading cells, in situ hybridization experiments were conducted to visualize the developmental appearance of the sequence at the cellular level. Although cryptdin mRNA has not been detected in Northern blots of intestinal RNA from 10-d-old mice (data not shown), in situ hybridization experiments show that cryptdin mRNA is present at a low level in
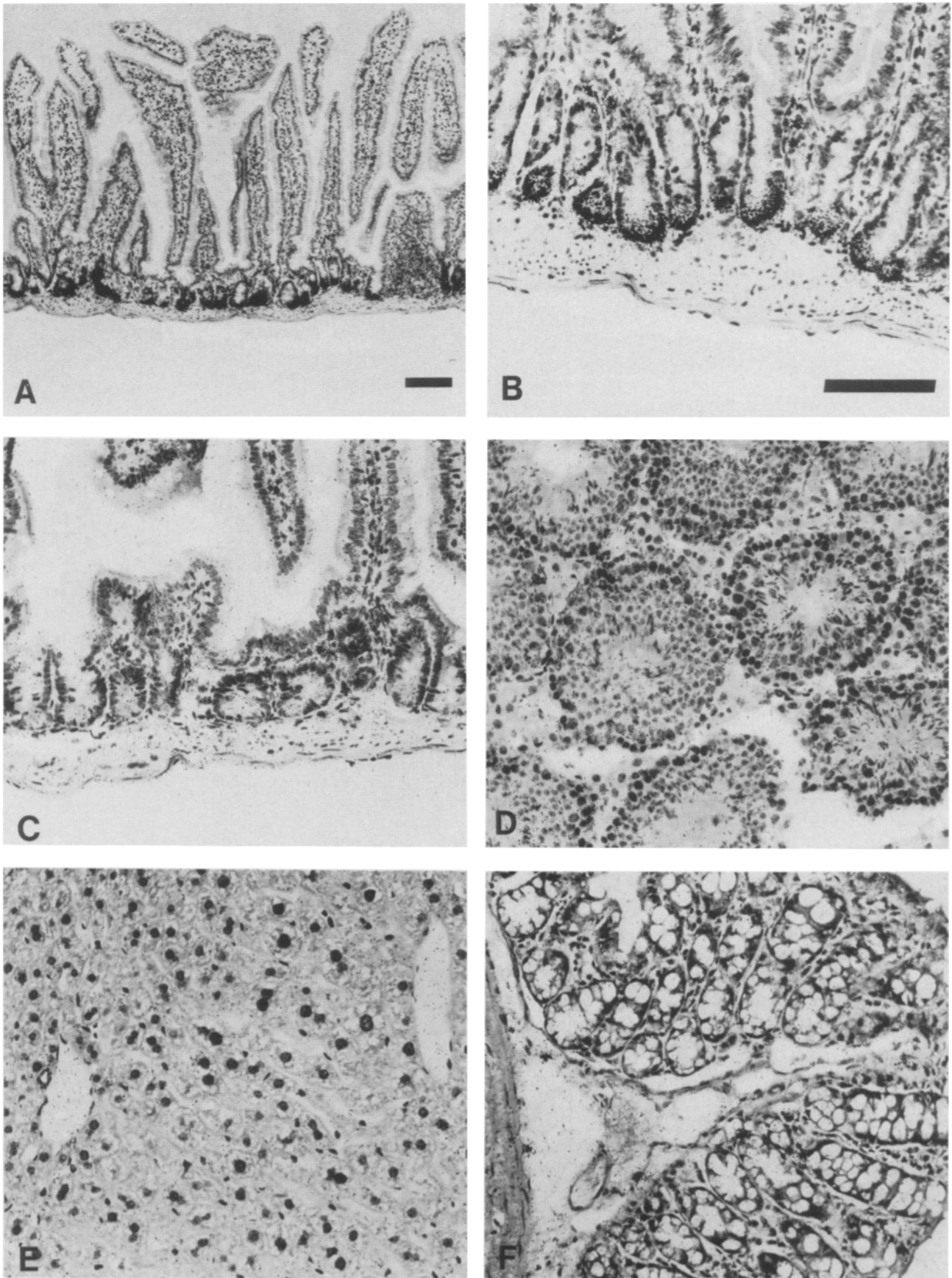
Figure 4. Cryptdin mRNA in crypt epithelium of adult mouse small intestine. Cryptdin cDNA insert labeled with [³⁵S]dCTP was hybridized to sections of adult mouse organs (see Materials and Methods). *A*, jejunum with 100,000 cpm applied to slide; *B*, jejunum with 20,000 cpm applied to slide; *C*, jejunum 300,000 cpm of ³⁵S-labeled pBR322 DNA per slide; *D*, testis with 100,000 cpm per slide; *E*, liver with 100,000 cpm per slide; *F*, colon with 100,000 cpm per slide. Sections shown in *B*, *D*, and *E* were hybridized on the same slide. Bars, 100 μm.
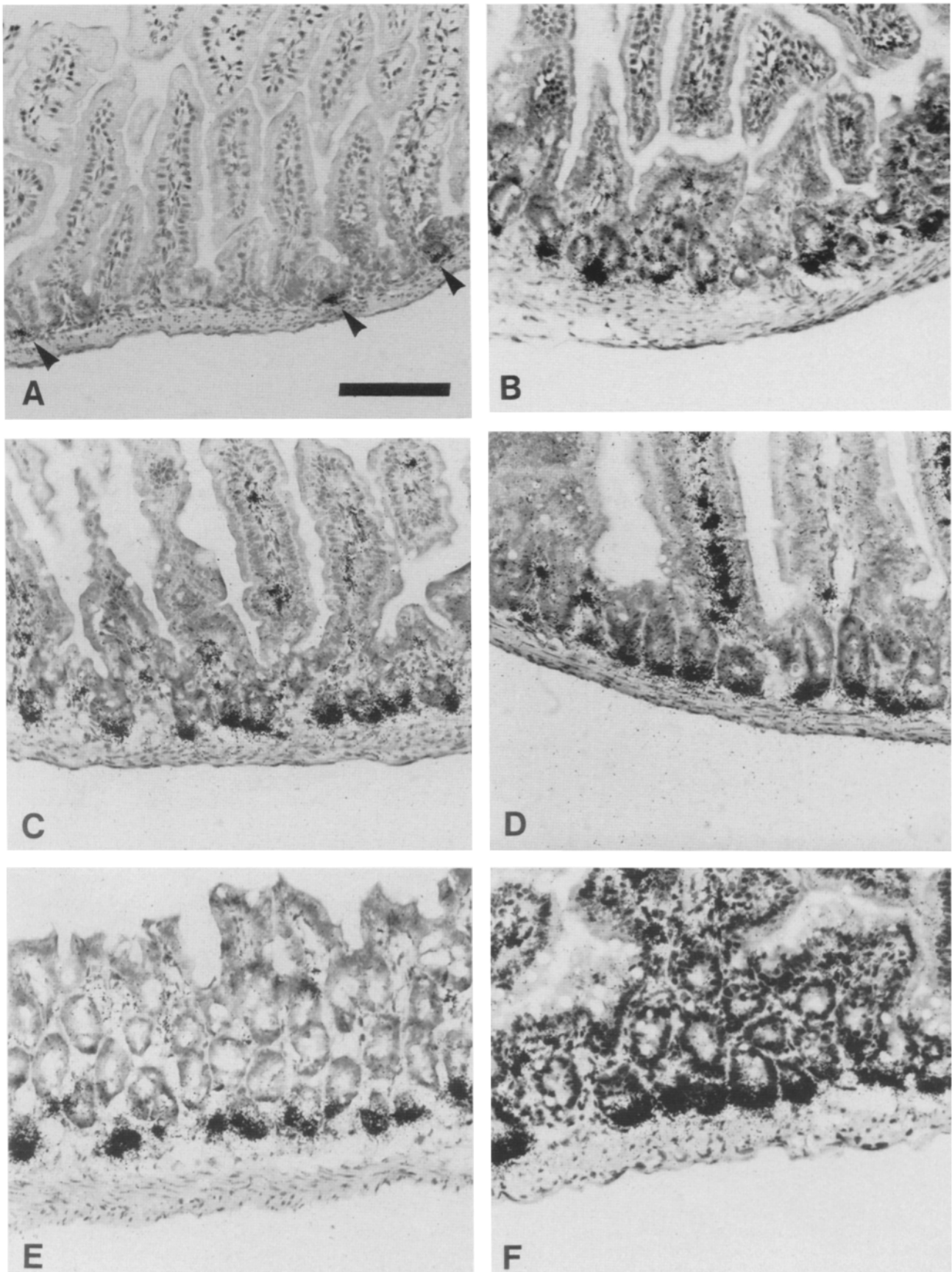
Figure 5. Developmental appearance of cryptdin mRNA in mouse intestinal crypts. A [³⁵S]dCTP-labeled cryptdin cDNA probe (50,000 cpm/slide) labeled by nick translation (Rigby et al., 1977) was applied to sections of mouse small bowel. Sections were from 10- (*A*), 16- (*B*), 20- (*C*), 24- (*D*), 30- (*E*), and 40- (*F*) d-old mice. Arrows in *A* denote positive crypts. Note positive hybridization to lamina propria leukocytes in *C* and *D*. Autoradiographs were exposed for 5 d. Bar, 100 μm.

5–10% of crypts in 10-d-olds (Fig. 5 A). By 16 d of age, 70–80% of small intestinal crypts hybridize positively and with greater intensity than those of 10-d-old mice (Fig. 5 B). By day 20, all crypts are positive and more intensely labeled with the cryptdin cDNA probe (Fig. 5 C). During the remainder of postnatal development, cryptdin mRNA content increases to the maximal adult levels in all crypts (Fig. 5, D–F). Cryptdin mRNA has not been measured in aged mice, but 4–6-mo-old mice have the same cryptdin mRNA content as younger adults (unpublished data). The kinetics and anatomy of cryptdin mRNA appearance in crypt epithelium are characteristic of Paneth development and consistent with the abundance of cryptdin mRNA in Paneth cells in the adult.

These results demonstrate that the cryptdin gene is induced in resident cells in the small bowel rather than being introduced by cells that migrate postnatally into the intestine. Because Paneth cells have been implicated in controlling mucosal microbes (Erlandsen and Chase, 1972) and differ morphologically in germ-free and naturally reared rodents (Satoh et al., 1986; Satoh and Vollrath, 1986), and because the cryptdin gene may be induced by local "factors" presented to individual crypts, the influence of luminal bacterial antigens and T cell–associated mediators were tested on cryptdin mRNA content in adult small bowel.

Cryptdin mRNA in Germ-free Mice

Cryptdin mRNA levels were measured in intestinal RNA from 28-d-old germ-free mice and from naturally reared mice. Its apparent localization in Paneth cells and its structural relation to antiviral/microbicidal defensins suggested that cryptdin could be sensitive to regulation by bacterial antigens. The gastrointestinal tracts of gnotobiotic mice were judged to be free of bacteria by inoculation of thioglycollate
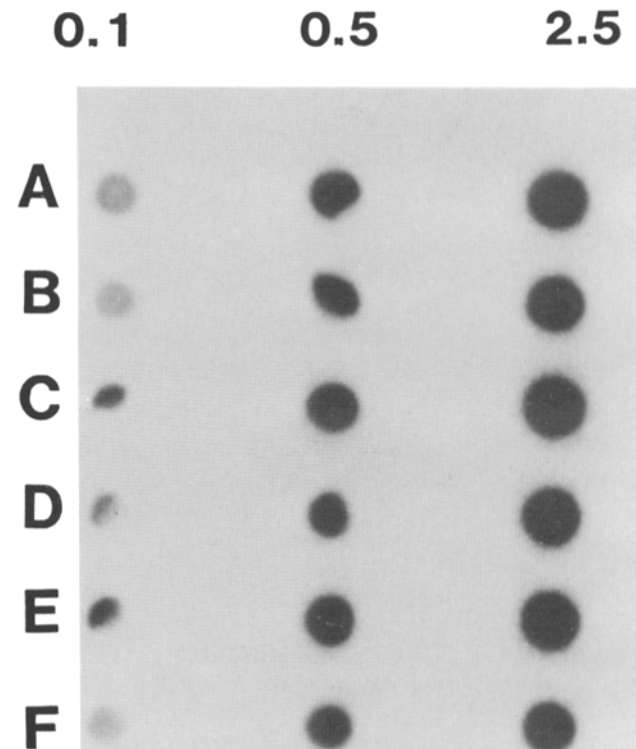
and LB broth with cecal contents; all germ-free mice were negative for both aerobes and anaerobes. As shown in Fig. 6, cryptdin mRNA is equally abundant in intestinal RNA from germ-free mice and mice reared naturally. Accordingly, bacterial-associated proteins or microbially produced chemotactic peptides (Marasco et al., 1984) appear to be excluded as determinants of cryptdin mRNA content in the adult small intestine. However, the presence of bacterial lipopolysaccharides or antigens from bacterial remnants in the autoclaved chow that nourished these mice cannot be excluded as potential inducers of the cryptdin gene. Nevertheless, live microorganisms appear not to influence intestinal cryptdin mRNA content.

Cryptdin mRNA in Athymic Mice

To test whether cryptdin gene induction is T cell dependent, cryptdin mRNA levels were measured in adult nude mice by RNA blot analysis. Cryptdin mRNA is equally abundant in full-length, full-thickness small intestine of the nude mouse and of the normal, outbred Swiss albino mouse used throughout these studies (Fig. 7). Accordingly, we conclude that the level of cryptdin mRNA in crypt epithelium of adult mice is independent of direct or indirect T cell mediation.

Discussion

Cryptdin is one of many low molecular weight mRNAs that accumulate in mouse small intestine during postnatal devel-

Figure 6. Cryptdin mRNA in small bowel of germ-free mice. Samples (10 µg) of total RNA isolated from full-length, full-thickness small intestine from three individual germ-free (lanes A–C) and 3 naturally reared (lanes D–F) mice were blotted and hybridized to cryptdin cDNA (see Materials and Methods). Autoradiogram was exposed for 4 h. Upper and lower bars at left denote 28S and 18S rRNA markers, respectively.

Figure 7. Cryptdin mRNA in small bowel of athymic mice. Small intestinal total RNA from three individual athymic (nu/nu) Swiss (A, C, and E) and from three outbred Swiss (B, D, and F) mice was applied in the quantities indicated, and the blot was hybridized with cryptdin cDNA (see Materials and Methods). Autoradiogram was exposed for 4 h.

opment and code for 6-kD cysteine-rich polypeptides (Birkenmeier and Gordon, 1986; Ouellette and Cordell, 1988). CS/defensins isolated from all sources contain a cysteine- and arginine-rich consensus sequence that confers a beta pleated sheet structure on these related proteins (Bach et al., 1987; Stanfield et al., 1988; Westbrook et al., 1984). Before the identification of intestinal cryptdin, CS/defensins had been found only in cells of myeloid origin (Selsted et al., 1985a,b; Zhu et al., 1988, 1989; Daher et al., 1988). Expression of this CS/defensin-related gene in intestinal epithelium, and its presence in adult testis and brain, provides an opportunity to define broader physiologic functions for cryptdin and perhaps other CS/defensins in cells of nonmyeloid origin.

Since the CS/defensin consensus residues constitute one-third of the mass in these 32-34 amino acid proteins, overall sequence similarities between members of this family may conceal important determinants of functional specificity. Outside of the consensus sequence (Fig. 3 C), cryptdin shows little identity with CS/defensins. Since the motif has a conserved structural role (Bach et al., 1987; Stanfield et al., 1988; Westbrook et al., 1984), sequence divergence in apparent domains outside of the consensus (Fig. 1, regions A and B) may specify function for peptides in this family. Variability in both corticostatin and defensin specific activities must result from substitutions within these domains, and residues 5-9 and 19-29 must determine the function of cryptdin in mouse crypt epithelium.

Although the factors that regulate cryptdin gene expression are unknown, several potential mediators have been eliminated from consideration. Androgenic regulation is excluded, because the sequence is equally abundant in female and male mice (data not shown). Cryptdin mRNA levels in the adult small bowel are independent of luminal bacteria (Fig. 6) and T cell mediation (Fig. 7). Also, the cryptdin gene is insensitive to induction by glucocorticoids, since administration of dexamethasone to 8-10-d-old sucklings does not elicit precocious appearance of cryptdin mRNA even though sucrase-isomaltase induction occurs in classical fashion (Ouellette, A. J., and D. Ménard, unpublished data). Interestingly, accumulation of defensins is developmentally regulated in alveolar macrophages (Ganz et al., 1985a), suggesting the possibility of common regulatory elements in these genes.

What is the role of cryptdin in the small bowel? Circumstantial evidence supports a role for cryptdin in the regulation of intestinal microflora or in the inhibition of bacterial translocation. First, cryptdin is developmentally regulated in crypt Paneth cells which contain lysozyme (Erlandsen et al., 1974; Hammer et al., 1987) and have been implicated in controlling mucosal microorganisms (Elmes et al., 1984; Erlandsen and Chase, 1972). Second, as shown in Fig. 3, cryptdin shows highest sequence similarity to MCP-1 (SC III) and MCP-2 (CS IV), the most potent antiviral/microbicidal defensins (Ganz et al., 1985b; Lehrer et al., 1983). Third, MCP-1 and MCP-2 resemble cryptdin more closely than they resemble other CS/defensins, even those isolated from the same cells (Selsted et al., 1985a). Fourth, appearance of cryptdin mRNA in mouse small bowel coincides with gut closure and with the establishment of a barrier to bacterial translocation. Fifth, since mutants of Salmonella typhimurium exhibit differential sensitivity to MCP-1 and NP-5 defensins

(Groisman, E., personal communication), the existence of defensin targets and mechanisms of defensin resistance in enteric microorganisms seems likely. Furthermore, cryptdin mRNA is sufficiently abundant in the small bowel to support cryptdin synthesis in quantities required for defensin-like action in the lumen.

In spite of evidence supporting the concept of antiviral or antibacterial activities for cryptdin, alternatives to defensin action seem to be equally plausible and compelling. For example, defensins NP-3a, NP-3b, MCP-1, and MCP-2 (corticostatins I-IV, respectively) all inhibit ACTH-stimulated corticosteroid production to varying degrees in cultures of rat adrenal cells (Zhu et al., 1989). The inhibition occurs at concentrations of 10-50 ng/ml, in contrast to the 50-100 μg/ml quantities of these peptides required for defensin activity. Thus, we speculate that cryptdin may have corticostatic or analogous paracrine effects on the intestinal epithelium, a tissue that is highly responsive to glucocorticoids and to other hormones (Henning, 1985). The apparently dual functions of CS/defensins and the abundance of cryptdin mRNA in crypts populated both by Paneth and neuroendocrine cells, suggest that cryptdin may exhibit both defensin-like and corticostatin-like activities in the mouse small bowel.

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