The eosinophil-derived neurotoxin (EDN/RNS2) is a member of the mammalian ribonuclease gene family and is one of four proteins found in the large specific granules of human eosinophilic leukocytes. The gene encoding EDN consists of two exons, including a non-coding exon 1, separated by a single intron from the coding sequence in exon 2. We have identified a functional promoter of the EDN gene and shown that optimal expression depends on interaction between the promoter and one or more sequence elements found in the single intron. Cells of the clone 15 eosinophilic variant of the human promyelocytic HL-60 cell line were transfected with constructs that included the promoter region of the EDN gene alone, promoter with exon 1, and promoter with both exon 1 and the intron positioned 5′ to the chloramphenicol acetyltransferase (CAT) reporter gene (constructs referred to as PrCAT, PrExCAT, and PrExInCAT, respectively). Although reporter gene activity from either PrCAT or PrExCAT was only 2-3 fold higher than baseline (CAT alone), inclusion of the single intron (PrExInCAT) resulted in a 28-fold increase in reporter gene activity in uninduced clone 15 cells, and an 80-fold in activity when clone 15 cells were induced to differentiate toward eosinophils with butyric acid. The intron-mediated enhancer activity was reproduced in other human hematopoietic cell lines (K562, Jurkat, U937, and HL-60), but was not found in human 293 kidney cells, suggesting that the function of the enhancer element(s) may be tissue-specific. A significant portion of the observed enhancer activity resides in the first 60 base pairs of the intron, which includes consensus binding sites for both AP-1 and NF-ATp transcription factors, and a 15-base pair segment that is identical to a sequence found in the promoter of the gene encoding the neutrophil granule protein, lactoferrin. The noncoding exon 1/single intron/coding exon 2 genomic structure is a common feature among the mammalian ribonucleases; this finding suggests the possibility of a conserved mechanism of regulation in this gene family.

The regulation of gene expression is critical to the development of cells and organisms. Although the regulation of gene expression during hematopoiesis has been under intense scrutiny, few studies have examined the eosinophil lineage directly. Whereas the regulatory regions of several eosinophil genes have been characterized (1–4), the specific molecular events underlying commitment to and differentiation of the eosinophil lineage remain unknown.

The eosinophil-derived neurotoxin (EDN) is a small, cationic granule protein synthesized during the promyelocyte stage of eosinophil development (5, 6). The cDNA sequence and complete open reading frame identified EDN as a member of the ribonuclease gene family (7, 8). The gene encoding EDN (1.2 kilobases, designated RNS2) contains two exons separated by a 230-bp intron, with the entire coding sequence residing on exon 2 (9). This gene structure (noncoding exon 1/single intron/coding exon 2) is shared by at least three additional ribonuclease genes, including eosinophil cationic protein (ECP) (9), angiogenin (10, 11), and pancreatic ribonuclease (12, 13), and appears to be a consistent feature of this gene family.

The studies to be described were performed by transfection of various segments of the EDN gene into clone 15 cells; clone 15 is a subline of the human promyelocytic leukemia cell line, HL-60, which was isolated on the basis of its propensity to develop into cells resembling mature eosinophils (14). Features of this cell line include formation of Luxol-fast blue staining granules (14, 15), biosynthesis of eosinophil major basic protein and eosinophil peroxidase (15), and the ability to express cell surface receptors for interleukin-5 (16, 17). Most recently, we have shown that differentiated clone 15 cells also synthesize immunoreactive ECP and EDN (18).

In the work presented here, we have identified features within the EDN gene that are responsible for promoting gene expression in human cell lines, including at least one functional enhancer element within the characteristic single intron.

**EXPERIMENTAL PROCEDURES**

Cell Lines—The human cell lines used in this investigation were obtained from the American Type Tissue Culture Collection (Rockville, MD) and maintained as indicated. The HL-60, (promyelocytic leukemia), U937, (histiocytic lymphoma), Jurkat, (acute T cell leukemia), and K562 (chronic myelogenous leukemia) were all grown in RPMI 1640 medium (Biofluids, Inc., Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Inc, Logan, Utah or Life Technologies, Inc.), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at pH 7.2. The HL-60 clone 15 promyelocytic leukemia cell's were grown in the same medium supplemented with 0.1% nonessential amino acids (Biofluids, Inc.).

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¶ The abbreviations used are: EDN, eosinophil-derived neurotoxin; ECP, eosinophil cationic protein; bp, base pair(s); CAT, chloramphenicol acetyltransferase; HEPPSO, N-[2-hydroxyethyl]piperazine-N′-[2-hydroxypropanesulfonic acid; BA, butyric acid; MOPS, 3-morpholino propanesulfonic acid; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction.

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with 25 mM HEPPSO (Sigma) to maintain the pH at 7.6. The human 293 cells, a transformed primary embryonic kidney cell line, were grown in Dulbecco's modified Eagle's medium (Biofluids, Inc.) supplemented as above. All cells were grown at 37 °C and 5% carbon dioxide in a humidified incubator.

Cellular Differentiation—The clone 15 cells were induced to differentiate in a L-thionin neurite outgrowth line along with the addition of 0.5 mM butyric acid (BA) (Sigma) to freshly cultured cells at a concentration of 0.5 × 10^6 cells/ml as described by Fischkoff and colleagues (16, 17).

RNA Isolation and Northern Analysis—Total RNA was isolated at times indicated using the method of Chirgwin et al. (19) or using an RNA isolation kit (Stratagene, La Jolla, CA). Isolated RNA (15 μg/ml) was denatured in 1% agarose gel containing 2% formaldehyde (10 mM MOPS buffer, 5 mM sodium acetate, and 1 mM EDTA at pH 7.0). After electrophoresis, RNA was transferred overnight to a Hybond-N nylon filter (Amersham Corp.), UV-cross-linked using a Stratalinker (Stratagene), and probed with the following gene-specific antisense oligonucleotide probe sequences: (a) EDN, 5'-GTGACATTTTGCCTCATTTGTTACCATCGAACGAGCTATTTGGT-3', corresponding to bases 341–296 of EDN cDNA (7); (b) ECP, 5'-TGGCCGGATTGACTTCATCGCAGGAGGTTG-3', corresponding to bases 222–184 of ECP cDNA (20); and (c) human beta-actin, 5'-GGCAGCAGCGGCGGCTGATCAT-3', corresponding to amino acids 16–2 encoded by human beta actin (21). Fifty nanograms of each oligonucleotide probe were radiolabeled using T4 polynucleotide kinase (Promega) or [γ-32P]ATP (5000Ci/mmol, ICN, San Diego, CA) and purified on size exclusion columns (Stratagene). Nylon filters containing RNA were prehybridized (40% formamide, 10% dextran sulfate, 4× SSC, 20 mM HEPPS, 10 mM MOPS buffer, 5 mM sodium acetate, and 1 mM EDTA at pH 7.6. The human 293 cells, a transformed primary embryonic kidney cell line, were grown in a humidified incubator.

Rapid Amplification of cDNA Ends—Real-time PCR was performed by dot blotting using an oligonucleotide primer specific for EDN-1a (see Fig. 1B) and by dyeoxy sequencing. The nucleotide sequence obtained agreed with previously published data (9).

Reporter Gene Constructs—The reporter gene used in these studies was chloramphenicol-acetyltransferase (CAT) as found in the pCAT basic expression vector (Promega). Various fragments of the 5'-putative promoter region and with and without 5'-flanking sequence were amplified using the following primers (numbered as per Ref. 9): 1, 5'-CTGAGGAGGACATGATTTTGGC-3', nucleotides 312 to 288; 2, 5'-GGTCCCTCTTACTTGAAC-3', nucleotides 26 to 45; 3, 5'-CTGTAAGAAAAAGAAAGAGAATAC-3', nucleotides 172 to 149; 4, 5'-CCAGTTCGCGCTGAGC-3', nucleotides 42–43; 5, 5'-TGAATCAACATGGGCCCT-3', nucleotides 43–60; 6, 5'-CCCTCACGTTGCGGCCATC-3', nucleotides 102–82; 7, 5'-CTTACTCTCTGCAGGCGA-3', nucleotides 162–142; 8, 5'-ACACACAGTGAGTTCCAT-3', nucleotides 222–202. These primers also contained 5' PstI or XbaI sites as indicated to facilitate subcloning into the pCAT vectors. For the construct containing promoter plus intron, without exon 1, the primers used were 1 and 9 (nucleotides 26–45 with a 5' XhoI site) and pCMV-β-galactosidase vector to control for transfection efficiency. The level of β-galactosidase activity was determined spectrophotometrically (β-galactosidase assay kit from Promega Corp.) or using a Gene Pulser (Bio-Rad). The cells were plated on ice for 15 min prior to transfection to reduce heat shock. Transfection was performed using 40 μg of construct with the XhoI site and 3 were used to amplify the intron sequence. This produced a promoter construct with a XhoI site at its 3' and an intron construct with the XhoI site at its 5' end after PCR amplification. The products were gel purified, restriction digested with XhoI, and ligated; the ligation was followed by a second round of PCR amplification using primers 1 and 3 described above. The 520-bp fragment was gel purified and ligated into the pCAT-basic vector. All constructs were confirmed by dyeoxy sequencing.

Electroporation of CAT Constructs into Human Cell Lines—Cell lines as described above were grown to a density of 0.5 to 1.0 × 10^6 cells/ml. Cells were harvested by a 1-min centrifugation in a microfuge and resuspended in 0.5 ml of 40 mM Tris, pH 7.4, with 1 mM EDTA and 150 mM NaCl. Cells were then harvested by a 1-min centrifugation in a microfuge and resuspended in 100–150 μl of 25 mM Tris, pH 8, and disrupted by freeze-thaw, three times on dry ice/37 °C water bath. The debris was removed by centrifugation; clarified extract was added to the following reaction mixture: 5–45 μl of extract, 35 μl of 1× Tris, pH 7.5, 20 μl of 4 μM acetyl CoA (Pharmacia Biotech Inc.), and 3–5 μl of [14C]chloramphenicol (0.25 μCi, DuPont NEN), with distilled H_2O added to a final volume of 150 μl. After 6–12 h at 37 °C, the [14C]chloramphenicol and acetylated products were extracted by vortexing for 30 min with 1 ml of ethyl acetate. The ethyl acetate layer (upper) was removed and air dried under a hood or SpeedVac (Savant). The acetyl signal was measured using a System 200 Image Scanner (Bioscan, Inc., Washington, D.C.).

Analysis of Intrinsic Region—Computer analysis of the intrinsic regions of the EDN gene for consensus transcription factor binding sites was performed using the MacVector sequence analysis program. Community
comparision of intronic regions with known sequences was performed using the FASTA algorithm of the Wisconsin Genetics Computer Group program on-line at the National Institutes of Health.

Sequence Mutations—Specific point mutations were introduced into PrExIn by overlapping PCR mutagenesis (23) using the following oligonucleotide primer pairs along with primers 1 and 8 as described previously: lfn X (5′ primer, 5′-TCAACGCATCCCCTGGTGTCGCAAGGGGCCAGCAAT-3′, and 3′ primer, 5′-ATTGCTGCCCCCTTGCTTGCGACCCAGGGATCGTTGA-3′) and lfn Y (5′ primer, 5′-TCAACGCATCCCCTGGTGTCGCAAGGGGCCAGCAAT-3′, and 3′ primer, 5′-ATTGCTGCCCCCTTGCTTGCGACCCAGGGATCGTTGA-3′). The altered sequences were subcloned into pCAT basic as described. All mutations were confirmed by dideoxy sequencing.

RESULTS

RNA Expression in the Clone 15 Cell Line—Expression of mRNA for the EDN in the clone 15 cell line is demonstrated in Fig. 1A. Messenger RNA encoding EDN was detected in uninduced clone 15 cells (lanes 1–7); the level of mRNA accumulating in these cells increased 6–7-fold within 48 h after the addition of 0.5 mM BA (lane 9) as determined by densitometric analysis. Hybridization with a β-actin probe (below) demonstrated relative loading of each lane.

Primer Extension—Primer extension experiments were undertaken to determine the precise location of the transcriptional start site. RNA from clone 15 cells induced for 2 days with 0.5 mM BA was used for this analysis; a control RNA reaction was included, as was a known DNA sequence for determination of molecular size (Fig. 1C). The band in the +EDN mRNA lane indicated by the arrowhead is the reverse-transcribed product. Comparison of the nucleotides in the known sequence (Std) with the mobility of the band indicated in the +EDN mRNA lane demonstrates that the distance from the primer to the 5′ end of EDN is 219 nucleotides. This result suggests that there are 25 additional nucleotides in the mRNA sequence that had not previously been identified by cDNA cloning (7, 8).

Rapid Amplification of cDNA Ends—It was unclear as to whether these additional 25 nucleotides represented a direct extension of exon 1 or a completely distinct exon; RACE was undertaken to make this determination. Using the poly(A)-selected mRNA described for the primer extension experiments, a cDNA strand complementary to the 5′ region of the EDN mRNA was reverse transcribed using the EDN or EDN-2a primers (Fig. 1B), PCR amplified, and subcloned. Numerous colonies were obtained; 18 random colonies were selected for sequencing. The results from a representative sequence are depicted in Fig. 1D. The additional 25-base pair sequence obtained, which follows the anchor sequence and precedes the first exon sequence, is 5′-AGCTGGCGCCCGAAGGGGCACTGGGGATCGTTGA-3′. This sequence matches the published genomic sequence of Hamann and colleagues (9) that is found immediately 5′ to the previously designated exon 1. These results suggest that the additional nucleotides represent a direct extension of exon 1 as opposed to another distinct exon. The transcriptional start site defined by both primer extension and RACE is located 25 nucleotides 3′ from a consensus TATA box (9). Of the 18 clones, 11 sequences were as described in Fig. 1D, two were 4 nucleotides shorter at the 5′ end, and five contained unidentified sequences between the PCR primers. The five unidentified sequences are assumed to be the result of incorrect priming, because they contained no sequences representing either exon 1 or exon 2.

CAT Activity in Clone 15 Cells—The sequence of the EDN gene positioned 5′ to CAT used in the following experiments is shown in Fig. 2A. The EDN promoter region alone (PrCAT) supported a relatively low level of activity in clone 15 cells, representing 2–3-fold over that of the promoterless CAT (Fig. 2B). Surprisingly, the EDN promoter-exon-intron construct (PrExInCAT) supported significantly greater degree of reporter gene activity; 28-fold over CAT in uninduced clone 15 cells and 80-fold over CAT in clone 15 cells grown in the presence of BA (Fig. 2, B and C). The EDN promoter-exon (PrExCAT) construct produced low levels of CAT activity, similar to those of PrCAT alone, suggesting that the dramatic enhancement seen with PrExInCAT was due to sequence elements found in the

![Figure 1](image_url)

**Fig. 1.** A, Northern analysis of RNA isolated from the clone 15 eosinophilic variant of HL-60 grown for 0–5 days in the absence (lanes 1–6) or the presence (lanes 7–12) of 0.5 mM BA. Total RNA (10 μg/lane) was probed with a radiolabeled EDN-specific oligonucleotide (see "Experimental Procedures"); identical samples were probed with a radiolabeled human β-actin-specific oligonucleotide (see "Experimental Procedures") to demonstrate loading of each lane. B, structural schematic of the EDN gene (9). Transcribed sequences are shown as boxes. The black area depicts the open reading frame; the shaded areas represent untranslated transcript as previously documented by cDNA cloning (7, 8); the unshaded area indicates additional transcript demonstrated by primer extension and RACE (see C and D). Below, schematic depicting placement of oligonucleotide primers used in primer extension (EDN-1a) and RACE (EDN-2a and EDN) experiments as described under "Experimental Procedures." C, primer extension from EDN-1a (see B) from mRNA isolated from clone 15 cells induced for 2 days with 0.5 mM BA (A, lane 9) (EDN mRNA) and from control RNA; the + and − symbols indicate reactions performed with or without the addition of mRNA prior to reverse transcription. The primer extension product in the +EDN mRNA lane is indicated by the arrow; its migration relative to the flanking sequence standards (Std) indicates a 219-bp product, extending the known transcript size by 25 bp. D, sequence of the extended transcript isolated by the RACE procedure. Regions of known sequence are as designated (Anchor, Exon 1, Exon 2, and the ATG codon at the start of translation). Short arrows designate the sequence of the 25-bp extension to exon 1.
The intron alone (InCAT) did not demonstrate reporter gene activity above the basal levels in these cells, whereas the EDN promoter-intron combination (PrInCAT) without the first exon provided a 12-fold increase in activity over CAT alone. The increase in activity with promoter and intron and the absence of activity with the intron alone suggests that the enhanced activity depends on interactions between sequence elements in both the promoter and intron.

Expression of EDN-CAT Constructs in Other Cell Lines—Analysis of CAT activity in several other cell lines is shown in Fig. 3. PrExInCAT was capable of promoting reporter gene activity 20–40-fold over that observed with the CAT in the K562, Jurkat, U-937, and HL-60 human hematopoietic cell lines (Fig. 3). The EDN promoter alone (PrCAT) supported only the lower, basal levels of expression in all cell lines tested. To evaluate regulation in a nonhematopoietic cell line, the PrCAT and PrExInCAT constructs were introduced into the human transformed 293 kidney cell line. Similar to the observations made with the hematopoietic cells, PrCAT induced a basal (2–3-fold) increase in reporter gene activity over CAT in the human kidney cell line. Interestingly, in contrast to the 20–40-fold increase in activity observed in hematopoietic cells, PrExInCAT induced only a 3-fold increase in the kidney cells. This activity was not significantly greater than that induced by PrCAT, suggesting that the enhancer activity provided by the intron may function in a tissue-specific fashion.

Dissection and Analysis of the Intron—In order to define the region or regions of the intron that enhance gene expression, truncated promoter-intron constructs were evaluated in clone 15 cells (Fig. 4). The PrEx0.25InCAT and PrEx0.5InCAT constructs, which contain the first 60 and first 120 base pairs of the intron, respectively, were both found to increase reporter gene activity 8-fold over CAT alone. These results suggest that there are one or more functional enhancer elements in the first 60 base pairs of the intron. In contrast, the PrEx0.75InCAT construct, containing the first 180 bp of the intron, produces only a 2.5-fold increase in activity over CAT, suggesting that this region (120 and 180 bp) contains sequence elements that neutralize the effects of the aforementioned functional enhancer(s). Full activity (28-fold over CAT) is restored with the complete intron (PrExInCAT), suggesting the possibility of additional functional enhancers in this final segment of the intron.
A sequence map of the EDN gene with a focus on the intron is shown in Fig. 5A. The first 60 bp of the intron contain consensus binding sites for both AP-1 and NF-ATp transcription factors. In addition, there is a segment of 15 bp that is identical to a segment found in the promoter of the lactoferrin gene (see “Discussion”) (24). Point mutations were introduced into the sequence of this segment (Fig. 5B), and the PrExInCAT constructs both with and without mutations were evaluated for their ability to support reporter gene activity. Neither set of mutations (Ifn X or Ifn Y; Fig. 5, B and C) altered the intron-enhancing activity to any significant degree (21- and 35-fold over CAT alone, as compared with 28-fold for the wild type).

**DISCUSSION**

In the initial phase of this study, we determined the transcriptional start site of the EDN gene. Both primer extension and RACE extended the length of the mRNA to include an additional 25 nucleotides. This additional sequence matched the region of genomic sequence that was directly 5' to sequence identified as exon 1 (9). Thus, exon 1 has been enlarged to 67 nucleotides, and the two-exon structure of this gene is confirmed. The transcriptional start site is situated appropriately at 23 and 101 base pairs 3' to consensus TATA and CAAT boxes, respectively.

Although significantly more active than either PrCAT or PrExCAT, we found that PrInCAT was not as effective as PrExInCAT in producing reporter gene activity. It is possible that the noncoding exon 1, in conjunction with the intron, plays a specific role in regulating activity of the EDN gene. However, it is also possible that the spatial rearrangement caused by deletion of this 67-base pair segment affects the ability of promoter and intron binding proteins to interact with each other as they interact with the DNA. This point may be clarified once the binding proteins mediating transcription of the EDN gene have been identified. Furthermore, it is not clear whether the enhancer elements present in the EDN intron coordinate specifically with the EDN promoter, or whether they might function equally effectively with other unrelated gene sequences.
promoters. Although not completely independent (InCAT produced little reporter gene activity in clone 15 cells), the specificity of the interaction between the intron and the EDN promoter has not been established.

Investigators have been examining the promoter regions of granule protein genes (1–4, 24, 25) in hopes of identifying factors promoting their tissue-specific expression. Our initial results suggest that the function of one or more of the intronic enhancer elements may be tissue-specific. In contrast to the results obtained with the human hematopoietic cell lines, the PrExInCAT construct supported no additional reporter gene activity over that of the promoter alone (PrCAT) in the human kidney cell line. Although preliminary, the increase in activity of PrExInCAT in the hematopoietic cells but not in the kidney cell line suggests that one or more of the functional enhancer elements may interact with a transcription factor expressed in a limited range of human cell types.

Sequence analysis of the EDN intron revealed the presence of several consensus sequences for transcription factor binding (Fig. 5). In the first 60 bp of the intron, the region in which a large portion of the enhancing activity resides, there are consensus sequences representing binding sites for transcription factors AP-1 (26) and NF-ATp (27). In addition, a 15-base pair segment that is identical to a segment found in the 5' promoter region of the neutrophil-specific granule protein, lactoferrin (24), is highlighted. The coordinate control of eosinophil and neutrophil granule protein biosynthesis was suggested by a series of studies examining the rare genetic disorder known as neutrophil specific granule deficiency (28, 29). Lomax and colleagues (30) determined that the defective biosynthesis of lactoferrin observed in this disorder resulted from a defect in mRNA transcription affecting specifically cells of the neutrophil lineage. Rosenberg and Gallin (31) showed that the biosynthetic defect could extend to include eosinophils; EDN was among the eosinophil granule proteins affected. Specific mutations introduced into the lactoferrin site resulted in no significant alteration in reporter gene activity, suggesting that this segment is not crucial to this specific aspect of EDN gene regulation. Additional investigations using electrophoretic mobility shift assays coupled with DNA footprinting and mutational analysis of additional sites will be necessary to determine the source of the enhancing activity.

In summary, this investigation has demonstrated that the intron of the EDN gene contains one or more functional en-

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**Fig. 5.** A, nucleotide sequence of the 5' intron (68–297) and the beginning of exon 2 (298 onward). Consensus CAAT and TATA box promoter sequences are as indicated. Consensus transcription factor binding sites (AP-1, NF-ATp, and PU.1) are single overlined; the arrowheads denote the division points for PrExIn0.25CAT, PrExIn0.5InCAT, and PrExIn0.75InCAT, respectively. The 15-bp segment that is double overlined is identical to a segment found in the 5' promoter region of the neutrophil specific granule protein, lactoferrin (24). B, mutations introduced into the 15-bp lactoferrin segment in the intron to create lfn X and lfn Y in PrExIn. Points at which lfn X and lfn Y differ from the wild type are indicated over each sequence with filled circles. C, reporter gene activity of each construct transfected into clone 15 cells, relative to that of CAT (no promoter). The bars represent densitometric analysis of duplicate samples.

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hancer elements and that interaction between the promoter and these intrinsic elements is required for the optimal gene expression. Although intrinsic enhancer elements have been described previously (32–39), this is the first evidence that this mechanism exists within the mammalian ribonuclease gene family. This finding takes on considerable significance because the noncoding exon/single intron/coding exon gene structure is shared by all members of this gene family whose gene structures have been determined (9–13). Although the nucleotide sequences of introns of each of the characterized ribonuclease genes are not overtly homologous to one another (save for EDN and ECP, whose introns are virtually identical (9)), the potential for this as a shared mechanism of gene expression is intriguing.

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