Intronic Enhancer Activity of the Eosinophil-derived Neurotoxin (RNS2) and Eosinophil Cationic Protein (RNS3) Genes Is Mediated by an NFAT-1 Consensus Binding Sequence*

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The eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP) are both small, cationic ribonuclease toxins that are stored in and secreted by activated human eosinophilic leukocytes. We have previously shown that optimal expression of the EDN gene is dependent on an interaction between an intronic enhancer element or elements and the 5′ promoter region. Here we present evidence demonstrating that the gene encoding ECP is regulated in an analogous fashion and that an intronic enhancer element functioning in both genes is a consensus binding sequence for the transcription factor NFAT-1. Our initial results demonstrate that one or more nuclear proteins isolated from human promyelocytic leukemia (HL-60) cells bind specifically at this consensus site (5′-GGAGAG-3′) within the intron of the EDN gene and that disruption of this sequence reduced the characteristic 20–30-fold increase in reporter gene activity observed with the tandem EDN promoter/exon 1/intron construct to background levels. The NFAT-1 consensus site in the ECP gene differs from that found in the EDN gene by a single nucleotide (5′-GGAGAG-3′); the conversion of the 3′ G to an A resulted in a further enhancement of the reporter gene activity supported by the ECP promoter/exon 1/intron construct. Interestingly, no “supershift” was observed in gel shift assays performed in the presence of anti-NFAT-1 antiserum, suggesting that a nuclear protein other than NFAT-1 may be acting at this consensus site.

Eosinophil-derived neurotoxin (EDN1/RNS2) and eosinophil cationic protein (ECP/RNS3) are members of the mammalian ribonuclease (RNome A) superfamily and are two of four proteins found in the large specific granules of human eosinophilic leukocytes (1, 2). The genes encoding EDN and ECP are 90% homologous to one another and both include two exons; each gene contains a noncoding exon 1, separated by a single intron from the coding sequence in exon 2. This gene structure is characteristic of the ribonuclease gene family (3–6). In our previous work, we have demonstrated that optimal expression of the EDN gene is dependent on interaction between the 5′ promoter region and the single intron (7). Further analysis of the EDN gene demonstrated that a significant portion of this intron-mediated enhancer activity resided within the first 60 base pairs of the intron, which includes consensus binding sites for both AP-1 and NFAT-1 transcription factors (7).

The NFAT-1 sequence is a consensus binding site for nuclear factor of activated T cells, preexisting (formerly known as NFATp), which was originally described as a cyclosporin-sensitive T lymphocyte-specific transcription factor (8–11) involved in the regulation of gene expression of several cytokine genes, including murine interleukin-2 (8), interleukin-3 (12), interleukin-4 (13–16), interleukin-5 (17), gp39 (18), granulocyte-macrophage colony-stimulating factor (19, 20), and tumor necrosis factor α (21). NFAT-1 exists as a cytosolic protein that is dephosphorylated and translocated to the nucleus following T lymphocyte activation by a calcium/calcineurin dependent mechanism, where it interacts cooperatively with Fos-Jun dimers (11, 22, 23) to regulate gene expression. NFAT-1 is also involved in the regulation of expression of cytokine genes in B lymphocytes (24–26) and in mast cells (27) and of granulocyte-macrophage colony-stimulating factor in endothelial cells (12). NFAT-1 immunoreactivity has been observed in murine neuronal cell lines (28), suggesting an even wider role for this factor as a regulator of gene expression.

Here, we find that optimal expression of the ECP gene also depends on the presence of an intronic enhancer element, and we present evidence demonstrating a role for an NFAT-1 consensus sequence and its binding protein in the regulated expression of both EDN and ECP.

EXPERIMENTAL PROCEDURES

Cell Culture—All cell lines used in this investigation were obtained from the American Type Culture Collection (Rockville, MD). The HL-60 (human promyelocytic leukemia) and K-562 (human chronic myelogenous leukemia) cell lines were cultured in RPMI 1640 (Biofluids, Inc., Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT), 2 mM l-glutamine (Quality Biologicals, Inc., Gaithersburg, MD), and 100 units/ml penicillin plus 100 μg/ml streptomycin (Quality Biologicals). The HL-60 clone 15 promyelocytic leukemia cells were grown in RPMI 1640 with fetal bovine serum, 1-glutamine, penicillin/streptomycin supplemented with 25 mM HEPPSO (N-[2-hydroxyethyl]piperazine-N′-[2-hydroxypropanesulfonic acid] (Sigma), and maintained at pH 7.6. All cells were grown at 37°C with 5% carbon dioxide in a humidified incubator.

Reporter Gene Constructs—The reporter gene used in these studies was chloramphenicol acetyltransferase (CAT) as found in the pCAT-basic plasmid vector (Promega, Madison, WI). Preparation of the EDN reporter gene constructs was as described previously (7); the construct EDN3-CAT described here is identical to that described as EDN-PrExCAT in the aforementioned publication. The ECP reporter gene constructs (see Fig. 2B) were prepared as described (7) from a bacteriophage with an ECP-encoding human genomic DNA insert; the transcriptional start site of the ECP gene was determined to be at the analogous site to the start site in the gene encoding EDN.3

Sequence Mutations—The NFAT-1 consensus sequence (nucleotides 126–131) found in the promoter of EDN was altered by overlapping

1 The abbreviations used are: EDN, eosinophil-derived neurotoxin; ECP, eosinophil cationic protein; CAT, chloramphenicol acetyltransferase.

2 K. D. Dyer, and H. F. Rosenberg, unpublished data.

3 H. L. Tiffany, and H. F. Rosenberg, unpublished data.
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mutagenesis (29) of EDN3-CAT, creating EDN3M-CAT (see Fig. 3A) using the following oligonucleotide primer pairs: A and D, and B and C. Primer A: 5'-CTG CAG GCA GCA TAT AGT TTT CAT-3', nucleotides ~312 to ~288; primer B, 5'-CTG TAA GAA AAG AAG AGA AGT AGT-3'; primer C: 5'-CTGCC ACG AGC TGA TGT TAG, nucleotides 111–146; and primer D: 5'-CTA AGC TCA GCT CTC GGA GAA CTC AGT TGC TGC CCC, nucleotides 146–111.

The NFAT-1 consensus sequence found in the promoter of EDN was introduced into the homologous site in the ECP promoter (nucleotides 126–131) also by overlapping mutagenesis (29) of ECP3-CAT, creating ECP3-CAT using the following oligonucleotide primers (E and F) paired with primers A and D, respectively, as described above. Primer E: 5'-AGG GCA GCA CCT GAG GGA GTG AGC TGA AGT TAG, nucleotides 111–146; and primer F: 5'-CTA ACT TCA GCT CAC TCC TCC CTC AGT TGC TGC CCT, nucleotides 146–111.

CAT Assay—Cells from the human cell lines indicated were grown to a density of 0.5 × 10^5 to 1 × 10^6 cells/ml harvested by centrifugation, washed, and resuspended at 30 × 10^6 cells/ml in growth medium as described above. Ten μg of uncut reporter gene construct along with 10 μg of uncut pCMV (cytomegalovirus)-β-galactosidase plasmid (Promega) were added to a 0.5–ml cell suspension in an electroporation cuvette (0.4-cm gap) (Bio-Rad). Electroporation, harvest, and β-galactosidase assays were as described previously (7).

Results

of Nuclear Proteins—Nuclear protein fractions were obtained from cells of the HL-60 human promyelocytic leukemia cell line for use in electrophoretic mobility shift assays as described below. Cells (1 × 10^6 in log phase growth) were harvested (2000 rpm for 5 min) and washed once in sterile, ice-cold phosphate-buffered saline without calcium or magnesium. The washed cells were resuspended in 1.25 ml of cold buffer containing 10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 0.3 μM succrose, 0.1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (Sigma), and protease inhibitors (Boehringer Mannheim) as follows: 50 μg/ml antipain dihydrochloride, 0.7 μg/ml pepstatin, 330 μg/ml phosphoramidon, 2 μg/ml aprotinin, 60 μg/ml chymostatin, 0.5 μg/ml leupeptin, 40 μg/ml bestatin, 10 μg/ml E-64, and 1 mg/ml Pefabloc. Resuspended cells were harvested and resuspended in 0.5 ml of the same buffer and lysed in with 15 strokes with a B type pestle in a Dounce homogenizer. The supernatant containing the cytosolic contents from the lysed cells was removed, and the nucleated proteins were resuspended in 0.3 ml of ice-cold buffer as described above, with the addition of 25% glycerol and 0.2 mM EDTA. The resuspended nuclei were shaken (gentle agitation) for 30 min at 4°C, and the resulting supernatant, harvested after centrifugation (12,000 rpm for 15 min at 4°C), was dialyzed in a 10-kDa pore size membrane (Spectrum Laboratories, Houston, TX) at 4°C against more than 100 volumes of buffer containing 20 mM HEPES, pH 7.9, 20% glycerol, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride for 3–5 h. The dialyzed supernatant was clarified by centrifugation, and protein concentration was measured using the BCA protein assay reagent system (Pierce). Nuclear extracts were aliquoted and stored at −80°C.

Electrophoretic Mobility Shift Assay (EMSA)—Complementary oligonucleotides E and F as described above were hybridized by heating to 65°C, followed by gradual cooling (15 min) to 37°C. The 5’ ends of each strand were phosphorylated with 50 μCi [γ-32P]ATP (6000 Ci/mmol; DuPont NEN) and T4 polynucleotide kinase (Boehringer Mannheim), purified by phenol-chloroform extraction and redissolved in 10 μl Tris, pH 7.5, with 1 mM EDTA. One μl of the radiolabeled oligonucleotide probe was added to 17 μl of gel shift incubation buffer (Stratagene, La Jolla, CA) and 5 μl of a 1 μg/ml preparation of nuclear proteins described above (or distilled H2O as a negative control). Competing unlabeled oligonucleotide was added in 100-fold excess prior to the addition of the labeled probe. After a 30-min incubation at room temperature, 2 μl of 0.1% bromphenol blue dye was added, and 5 μl of each sample were evaluated by electrophoresis in a 6% polyacrylamide DNA retardation gel prepared with 0.5 × Tris-borate-EDTA (TBE) (Novex, San Diego, CA) with 0.25 × TBE running buffer at a constant 100V, 6–15 mA at room temperature.

Supershift analysis was performed using a polyclonal anti-NFAT-1 antibody raised against purified NFAT-1 protein by McCallf et al. (10, Uffrey et al. (10), Lake Placid, NY). EMSA was performed as described above; 1 μl of a 1:1000 dilution of the NFAT-1 antibody was added 10 min after addition of the probe to the nuclear extract and then allowed to incubate an additional 30 min. Positive control included the NFAT-1 recognition sequence described in Ref. 10 incubated with the same dilution of antisera and nuclear extract from Jurkat (human T lymphoma) cells, also as described (10).

RESULTS

Reporter Gene Activity/ECP—Shown in Fig. 1A is a comparison of the genomic sequences of EDN and ECP; exons 1 and 2 are demarcated by brackets. Also indicated are the sequences of probes and consensus sites that are described in the text.

Fig. 1B diagrams the regions of ECP gene used to create the reporter gene constructs. Segments including the 5’ promoter region (ECP1), with promoter exon 1 (ECP2) and promoter with exon 1 and intron (ECP3) were inserted 5’ to the CAT reporter gene. Various human hematopoietic cell lines were transfected with these constructs, and the ability of each to support reporter gene activity was measured. As shown in Fig. 1C, the ECP promoter (ECP1-CAT) and promoter with noncoding exon 1 (ECP2-CAT) supported only slightly more reporter gene activity than that supported by (promoterless) CAT alone in the HL-60 promyelocytic leukemia cell line (1.2-fold and 1.1-fold, respectively). Addition of the intron (ECP3-CAT) led to a 9-fold increase in reporter gene activity. Similar results were obtained in two additional cell lines tested; whereas ECP1-CAT or ECP2-CAT supported relatively small increases in activity, the activity of ECP3-CAT was measured at 8-fold and 18-fold over CAT in the HL-60 clone 15 and K-562 cell lines, respectively. These results suggest that optimal expression of the ECP gene depends on a sequence element (or elements) present in the single intron, analogous to, albeit less dramatic than, the results obtained previously for the gene encoding EDN (Ref. 7; Fig. 1C).

EMSA/NFAT-1 Consensus Binding Sequence within the Intron of the EDN Gene—We have shown previously that a significant portion of the intron-mediated enhancer activity of EDN resides in the first 60 base pairs (nucleotides 68–133) of the intron (7). In order to assess the presence of specific binding sites, EMSA or gel shift analysis was performed with nuclear proteins isolated from the promyelocytic leukemia HL-60 cells.

Nuclear proteins were incubated with radiolabeled double-stranded oligonucleotide probes c and d corresponding to nucleotides 68–103 and 104–137, respectively (Fig. 1A). As shown in Fig. 2A, two distinct bands migrating more slowly than the unbound probe (probe d) were detected after incubation with the HL-60 nuclear proteins. These bands could not be detected in incubations that included a 100-fold excess of unlabeled probe, suggesting recognition of a specific sequence element or elements by one or more nuclear proteins in this extract. Additionally, these bands could not be detected in incubations that included a double-stranded oligonucleotide that encoded the NFAT-1 consensus site (nucleotides 126–131). No competition was seen in the presence of a random 34-base pair double-stranded oligonucleotide or from oligonucleotide probes corresponding to any of the other sequences within the original probe (data not shown), suggesting the presence of a specific nuclear protein or proteins binding to the NFAT-1 consensus site (nucleotides 126–131). No specific binding was observed in gel shift experiments in which nuclear protein fractions were incubated with the double-stranded oligonucleotide probe c (nucleotides 68–103; data not shown).

In order to determine whether the protein binding to the consensus site could be identified as immunoreactive NFAT-1, supershift analysis was performed using a polyclonal antiserum raised against purified NFAT-1 protein (10). Although a supershift was observed using the anti-NFAT-1 antiserum in conjunction with the consensus site probe and nuclear extract described by McCallf et al. (10), no supershift was observed using the anti-NFAT-1 antiserum with the HL-60 nuclear proteins and probe d (Fig. 2B), suggesting the possibility that another protein, perhaps another member of the NFAT family...
of transcription factors, binds specifically to this intron-based consensus sequence.

**Reporter Gene Activity/Mutagenesis of the EDN and ECP Intronic NFAT-1 Consensus Sequences**—In order to determine the functional significance of this consensus site, each nucleotide was altered (5'-GGAGAA-3' to 5'-TTCTCC-3'), creating EDN3M-CAT (Fig. 3A). The HL-60, K562, and clone 15 cell lines were transfected with this construct, and the reporter gene activity was compared directly to that supported by EDN3-CAT and CAT alone. We have previously shown that EDN3-CAT supported reporter gene activity 20–30-fold over that of promoterless CAT alone in both the HL-60 and K562 cells. In the clone 15 cell line, disruption of the NFAT-1 site did not totally abolish reporter gene activity but reduced it to 50% of that observed for the EDN3-CAT construct with the consensus site intact.

Interestingly, the NFAT-1 consensus site in the analogous site in the ECP gene differs by a single nucleotide, with a G, rather than an A, at the 3' end (see Fig. 1A). To evaluate the role played by this single base pair change in this apparently crucial site, a 3' G to A conversion was introduced into the consensus site encoded by the ECP intron, creating ECP3A-CAT (Fig. 3A). The HL-60, K562, and clone 15 cell lines were transfected with this construct, and the reporter gene activity was compared directly to that supported by ECP3-CAT and CAT alone. We have previously shown that ECP3-CAT supported reporter gene activity 9-fold over that of the promoterless CAT (Fig. 1C); as shown in Fig. 3C, the reporter gene activity of the altered construct was significantly greater than that of ECP3-CAT, from 15- and 17-fold over CAT in the HL-60 and K562 cell lines, respectively, to 28-fold over CAT in clone 15, similar to the levels of enhancement observed for EDN3-CAT in this cell line.

**DISCUSSION**

In our previous work we showed that the presence of the single intron was necessary for optimal expression of the gene encoding the eosinophil ribonuclease EDN (7). In this work, we have shown that the gene encoding the eosinophil ribonuclease ECP is regulated in an analogous fashion and that a consensus binding sequence for the transcription factor NFAT-1 (8–11) found in the introns of both EDN and ECP genes play a crucial role in enhancing their expression. Although originally described as specific to T lymphocytes, NFAT-1 has been found to participate in transcriptional regulation in mast cells, endothelial cells, and B lymphocytes (12, 24–27). This work suggests the possibility of a role for NFAT-1 and/or related proteins (30, 31) in the transcriptional control of genes expressed in human granulocytes. Interestingly, whereas the NFAT-1 sequence accounts for the entire intron-mediated enhancer activity found in both the promyelocytic HL-60 cells and the chronic myelog-
En passant, to express (32), lactoferrin (33), Charcot-Leyden crystal protein (34), and major basic protein (35); this consensus sequence may have a wider role in the regulation of gene expression in the granulocyte lineages than has been previously appreciated. Particularly interesting is the potential relationship between this consensus sequence and the rare genetic disease, neutrophil-specific granule deficiency, which is a disorder of transcription of granule proteins affecting both the neutrophil and eosinophilic granulocyte lineages (36, 37). Although NFAT-1 is expressed in a variety of tissues, the results of the supershift analysis suggest a different identity for this protein; it remains to be seen whether this consensus sequence binding protein is a variant of NFAT-1, a member of the NFAT family of transcription factors (34, 35), or something completely unique. Future studies will seek to identify this protein, to determine its role in the regulation of EDN and ECP gene expression, and to assess its involvement in the physiology and pathophysiology of the human granulocyte lineages.
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