Cloning and Characterization of the Human Neutrophil-activating Peptide (ENA-78) Gene*

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The neutrophil-activating peptide (ENA-78) is an inflammatory chemokine which is produced concomitantly with interleukin-8 (IL-8) in response to stimulation with either interleukin-1 (IL-1β) or tumor necrosis factor-α (TNF-α). We have identified a full-length ENA-78 cDNA and isolated its genomic clone.

The gene was found to consist of four exons and three introns and its structure resembles the IL-8 gene. The human ENA-78 gene was mapped to chromosome 4q13-q21, the same locus as several other inflammatory cytokine genes. The transcription initiation site was mapped to a position 624 base pairs (bp) upstream from the translation initiation site. A fusion gene containing 125 bp upstream of exon 1 linked to a luciferase reporter gene was expressed in the human embryonic 293 cell line. The expression of the reporter gene was induced by TNF-α, IL-1β, or phorbol 12-myristate 13-acetate. The 125-bp promoter region contained the cis-regulatory elements for enhancer binding protein-like factor (C/EBP) and the nuclear factor (NF-κB). Transfection of 293 cells with deletion mutants demonstrated that the NF-κB element, but not the C/EBP site, is sufficient for expression and induction by either TNF-α or IL-1β. In contrast, the IL-8 gene requires both elements. This report demonstrates that ENA-78 and IL-8 genes shared great similarity in genomic structure and chromosome location. However, these two genes may be regulated by distinct mechanisms.

Infiltration of neutrophil leukocytes from peripheral blood into tissue is a hallmark of acute inflammatory disease and is mediated by several chemotactic factors, including interleukin-8 (IL-8), GRO-α, GRO-β, GRO-γ, and RANTES (1-6). This inflammatory chemokine family can be divided into two classes, termed CXC and CC subfamilies which are distinguished by whether the first 2 of 4 conserved cysteine residues are separated by an additional amino acid residue or adjacent to each other (1-8).

Human neutrophil-activating peptide (ENA-78) is a recently discovered molecule first identified in the conditioned media of the stimulated human pulmonary type II epithelial cell line A549. (9). It was produced and secreted concomitantly with IL-8, GRO-α, and GRO-γ in response to stimulation with either tumor necrosis factor (TNF-α) or interleukin-1 (IL-1β). ENA-78 has 78 amino acids and contains 4 cysteines positioned identically to other inflammatory cytokines such as IL-8 and belongs to the CXC subfamily of the inflammatory cytokines. Like other inflammatory cytokines, ENA-78 stimulates neutrophil-directed chemotaxis and increases the intracellular level of free calcium. Cross-densitization experiments suggested that ENA-78 may act through the same type of receptor as IL-8 and GROα (9).

The ENA-78 protein has been purified from the conditioned media of induced A549 cells. Based on the amino acid sequence of the mature protein, the partial cDNA sequences were obtained by reverse-transcription and PCR amplification of the message from A549 cells (9). Here we report the identification of the full-length cDNA and the isolation of the genomic clone. All the genes of the CXC subfamily discovered so far have been mapped to chromosome 4q13-q21 (4, 10), including the gene encoding for β-thromboglobulin and platelet factor-4 which are expressed by megakaryocytes in a tissue-specific fashion (11, 12). In this study, we also mapped the ENA-78 gene to chromosome 4q13-q21, in proximity to other members of the family.

The induction of IL-8 gene expression requires two cis-regulatory elements, one is the binding site for nuclear factor (NF-κB), and the other is the binding site for enhancer binding protein-like (C/EBP) factor (2, 13). Mutation in either element abolishes IL-1β, TNF-α, or PMA responsiveness of the IL-8 gene, suggesting that these two distinct cis elements are required and cooperate in the induction of gene expression by these three stimuli (14-16). To understand whether ENA-78 gene regulation is similar to that of IL-8, we constructed several deletion mutants and studied the effects of these two elements on ENA-78 gene regulation.

MATERIALS AND METHODS

Cell Culture—A549 pulmonary epithelial cells (American Type Culture Collection, Rockville, MD) were grown with RPMI 1640 medium with 10% fetal calf serum. For induction of ENA-78, confluent monolayers were washed with RPMI without fetal calf serum and stimulated for 24 h with 20 ng/ml TNF-α or 10 ng/ml IL-1β. Poly(A)+ RNA was extracted from the monolayer after 24 h of induction.

Identification of cDNA Clone—The 5'-end of cDNA clone was isolated by using the 5'-end rapid amplification cDNA end (RACE) system (Life Technologies, Inc.). Poly(A)+ RNA isolated from induced A549 cells was reverse-transcribed using a specific primer for ENA-78 (5'-CTGAT-CAAGACAAATTCCTC-3'). The first strand cDNA was tailed at the 3'-end with terminal deoxynucleotidyl transerase. The anchor primer (Life Technologies, Inc.) and a nested specific primer (5'-GCTACCACT- TCCACCTTGAG-3') were used as sense and antisense primers, respectively, to PCR amplify the 5'-end of the cDNA. The amplified 450-bp fragment was directly sequenced with the 373A DNA sequencer (Applied Biosystems, Inc.).

PCR Analysis of Chromosome Location of ENA-78 Gene—A panel of 23 monochromosomal DNA isolated from human rodent somatic cell hybrids purchased from Coriell Cell Repositio (Camden, NJ) was used as the template for PCR analysis to determine the chromosome location of the gene. The two primers (sense primer 5'-CTGTOGGTTACAGAC- CACCGAG-3' and antisense primer 5'-GCGCAAGAGACAAATTC-
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CTTC-3') were used in PCR to amplify a 220-bp fragment.

Isolation of the Genomic Clone—A library of 1 × 10^6 plaque-forming units from the chromosome 4 genomic library purchased from American Type Culture Collection was screened with a radioactive probe of mixture of three 22-nucleotide oligonucleotides encoding for ENA-78. These three oligonucleotides were 5'-CTTTGTTTAGGACCACAGC-3', 5'-GGAGGGAGTCTACACGACG-3', and 5'-GGGAATTCATCCCAAAATGATCA-3'. The phage DNA was prepared as described previously (17). A-Phage DNA was used as a template in determining DNA sequences.

Chromosomal in Situ Hybridization—A genomic clone A1 λ-DNA was labeled with digoxigenin-d-UTP and combined with sheared human DNA and hybridized to human metaphase chromosomes derived from phytohemagglutinin-stimulated peripheral blood lymphocytes in a solution containing 50% formamide, 10% dextran sulfate, and 2x SSC (1× SSC in 0.15 M NaCl and 15 mM sodium citrate, pH 7.0). Specific signal was detected with digoxigenin fluorescein isothiocyanate. Chromosomes were then counterstained with propidium iodide and analyzed.

Construction of Fusion Genes—Regions upstream of exon 1 of the ENA-78 gene were amplified by PCR from the phage DNA of genomic clone A1. Two primers (5'-CAACCTCTTGTCTGAGGACTCT- and 5'-ATGGAGGAGGATCCGGAGCACTC-) with NheI site in the sense primer and BamHI site in the antisense primer were used to amplify the 140-bp fragment (nucleotide -125 to nucleotide +15) of the promoter region. This fragment was cloned into the Nhel-BglII site of the PGL2 enhancer plasmid vector containing the entire coding sequences of firefly luciferase along with SV40 enhancer (Promega Corp., Madison, WI) to generate a fusion gene. To delete one of the cis-regulatory elements, the primer containing the antisense primer was synthesized with a 140-bp fragment with six nucleotides deleted and used in PCR amplification of the promoter region. To construct a mutant with three copies of cis-regulatory element, a sense primer containing three copies of the cis element sequences was similarly used in PCR amplification of the promoter region. This mutated fragment was similarly subcloned into the PGL2-enhancer vector as described above.

Transfection and Luciferase Activity Assay—The fusion genes were used along with the promotorless PGL2 enhancer plasmid DNA in the transfection of the human embryonic kidney 293 cells. Cells at density of 5 × 10^5/ml in a six-well plate were transfected with 2 μg of plasmid DNA using 20 μl of lipofectAMINE reagent (Life Technologies, Inc.). Forty-eight h after transfection, the medium was replaced with fresh medium with the addition of 100 ng/ml IL-1β or 100 ng/ml TNF-α or 100 ng/ml PMA. After stimulation with IL-1β or TNF-α or PMA for 24 h, the cells were collected and the luciferase activity was analyzed according to the protocol of the luciferase assay system (Promega Corp., Madison, WI).

RESULTS

Identification of Full-length cDNA—To identify the 5'-end of the ENA-78 cDNA, we used the RACE system to amplify the 5'-end sequence from A549 cells induced with TNF-α and IL-1β. The nucleotide sequences of the 5'-end are shown together with genomic sequences in Fig. 1. In the 5' terminus, four nucleotide discrepancies were observed (nucleotides 363, 365, 368, and 374) between our cDNA and the published sequences (9), although the encoded amino acid sequences are identical. Since our cDNA sequences were identical to the genomic clone sequences (see below), the discrepancies in the published cDNA sequences are probably due to the different codon usage in the degenerative oligonucleotides used in PCR amplification of the gene. The 3'-end cDNA was obtained by reverse transcription and PCR based on the 3'-end noncoding sequences of the genomic clone. At the 3'-end there were another six nucleotide sequence discrepancies between our cDNA and the published sequence; however, the encoded amino acids were not affected. These six nucleotides were located at nucleotides 658, 659, 661, 1023, 1026, and 1032. The reason for this could be the same as the 5'-end discrepancies. These 10 nucleotide sequences are indicated in Fig. 1. The full-length ENA-78 cDNA encodes a predicted polypeptide of 114 amino acids. The translation initiation codon was assigned to the nucleotide +97. Termination codons are present in all three reading frames upstream of this translation initiation codon. According to the patterns of amino acids near the signal sequence cleavage site (18), the potential cleavage site of signal peptide is located between serine and leucine (amino acids 17 and 18).

Genomic Structure of ENA-78—To isolate the ENA-78 genomic clone, we took the approach of screening a chromosome-specific library. By PCR analysis of DNA from a panel of human-rodent cell hybrids, we detected that the ENA-78 gene was located on chromosome 4. A library of 1 × 10^6 plaque-forming units recombinant phage from chromosome 4 genomic DNA was screened with a radioactive probe consisting of a mixture of three oligonucleotides encoding various region of ENA-78. Seventy clones were isolated. Clone A1 contained an insert of 11 kilobases whose nucleotide sequence was determined. The sequence of a total of 2180 nucleotides is shown in Fig. 1. The gene consisted of four exons and three introns. The sequences of the exon-intron junctions conform to the consensus sequence of the eukaryotic splice junction. The ENA-78 genomic structure is very similar to that of IL-8 (19). Comparison of genomic structures of the inflammatory cytokines ENA-78, IL-8, GRO-α, β, γ, platelet factor-4, and β-thromboglobulin (1, 19–21) is shown in Fig. 2. These inflammatory cytokine genes share the same size of exons 2, 3, and 4. Additionally, the conserved cysteines were located on exon 2 and exon 3 in all of these genes.

Determination of the Transcriptional Start Site—To determine the transcription start site of the human ENA-78 gene, primer extension was modified by using the 3'-end RACE. Two different primers shown in Fig. 1, complementary to nucleotides 92–115 and nucleotides 152–175, were used in reverse transcription of mRNA prepared from the induced A549 cells. After reverse transcription, the nested further 3'-primers complementary to nucleotides 68–91 and nucleotides 128–151 were used along with the anchor primer in PCR to amplify the 5'-end of the cDNA region. The amplified PCR fragments were directly sequenced. The nucleotide sequence demonstrated that both fragments contained an identical 5'-end. This cDNA end is also consistent with the result in the original identification of the full-length cDNA in which the primer complementary to nucleotides 602–625 was used in reverse transcription. Taken together, all three primers reverse-transcribed to the identical end of the cDNA. The transcription start site was determined and assigned as nucleotide +1 in Fig. 1.

Chromosome in Situ Hybridization—To further regionally localize the gene on chromosome 4, the phage DNA of the genomic clone was labeled with digoxigenin-d-UTP and then hybridized to human metaphase chromosomes. Specific labeling was detected on the proximal long arm of a group B chromosome. In order to confirm the identity of the specifically labeled chromosome, two additional experiments were conducted in which probes specific for the centromeres of chromosomes 4 and 5 were cohybridized with clone A1. These experiments clearly demonstrated that clone A1 is located on the proximal long arm of chromosome 4. Measurement of 10 specifically hybridized chromosome 4s showed that clone A1 maps to a position which is 10% of the distance from the centromere to the telomere of chromosome arm 4q. This area corresponds to the boundary between 4q13 and 4q21 (Fig. 3). A total of 75 metaphase cells were analyzed. Sixty-four cells exhibited specific signal. Thus, ENA-78 was mapped to 4q13–q21, the same locus as other members of the CXC subfamily of inflammatory cytokines including IL-8, GRO-α, GRO-β, and GRO-γ.

Identification of Potential Regulatory Elements—Upstream of exon 1 of the ENA-78 gene, there is an AT-rich region at position −31 bp which may act as a TATA box. Several other potential regulatory elements are present in this 5'-flanking region. These regulatory elements include AP-2 at nucleotide
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-135 bp (CCCCCCTCCC), NF-κB at nucleotide -90 bp (GG-GAAATTTC), and C/EBP at nucleotide -113 bp (TTCCACAC). In contrast to the IL-8 gene where the NF-κB and C/EBP regulatory elements are adjacent to each other, these two elements are separated by 16 bp in the ENA-78 gene.

In the 3'-untranslated region of the ENA-78 gene, there were four copies of the ATT1 sequence, which is frequently found in mRNA for other transiently expressed cytokines and which appears to be involved in mRNA degradation (22). No consensus polyadenylation signal was found in the 3'-untranslated

Fig. 1. The nucleotide sequence of genomic DNA of ENA-78. The amino acid sequences of the coding region are also shown. Nucleotides are numbered starting at the beginning of the exon 1. The potential TATA box is boxed. The discrepancies between this sequence and the previously published sequence (9) are indicated by dots. The cis-regulatory elements are underlined. The sequences involved in mRNA degradation are also underlined. The primers used in reverse transcription in the RACE system to determine transcription initiation sites (+1) are overlined. The boundaries of exons and introns are indicated by arrows.
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FIG. 2. Comparison of genomic structures of the inflammatory cytokine genes of the CXC family. Schematic representation of the various members of the CXC family which have a striking similarity in the genomic structure is shown. Boxes represent exons. 3'-Untranslated exon sequences are not shown. Amino acid residues encoded by each individual exon are indicated above the exon. Conserved cysteines (CXC and C) are also shown in the exon.

Gene Regulation of ENA-78 by TNF-α, IL-1β, and PMA—Production of ENA-78 in A549 cells was induced concomitantly with IL-8 in response to stimulation with TNF-α, IL-1β, or PMA. To characterize the DNA sequences involved in the ENA-78 gene expression induced by TNF-α or IL-1β or PMA, we used PCR to amplify five potential promoter fragments from the 5'-flanking region which contain different regulatory elements (Fig. 4A). This potential promoter region was subcloned into the PGL-2 enhancer vector containing the luciferase gene along with the SV40 enhancer to generate a fusion gene. The fusion genes were transfected into the human embryonic kidney 293 cell lines. From reverse transcription and PCR analysis, we were able to detect the ENA-78 message in 293 cells (data not shown). After transfection of the 293 cells with the fusion gene, the cells were stimulated for 24 h with TNF-α, IL-1β, or PMA, and the luciferase activities were analyzed. As shown in Fig. 4B, the 125 base pairs upstream of exon 1 contained in the pA fusion gene functioned as an active promoter for the downstream reporter gene in 293 cells, and the activity can be induced by IL-1β, TNF-α, or PMA at the level of a 2-3-fold increase compared to the uninduced cells. The fusion genes containing only the C/EBP (pC) or three copies of C/EBP (pB) showed very little luciferase activity. In contrast, the fusion gene pA containing only the NF-κB element showed luciferase activity similar to the pA fusion gene containing both C/EBP and NF-κB elements. Furthermore, the activity can be induced by the three stimuli. The results suggest that the C/EBP element is not required in the 293 cells for induction of the ENA-78 gene, and the NF-κB element alone is sufficient to function as a promoter and to respond to the induction by IL-1β or TNF-α, or PMA. In contrast, IL-8 requires both elements for gene induction (14). Consequently, the ENA-78 gene appears to be regulated differently from the IL-8 gene.

DISCUSSION

We have isolated the full-length cDNA and a genomic clone of the human ENA-78 gene. Comparing our cDNA sequence with the genomic sequence, we showed that there were 10 nucleotide sequence errors in the previously published cDNA sequence (9). However, these differences did not affect the predicted amino acid sequence of ENA-78. These 10 discrepancies may be caused by the degenerative primers used in the PCR amplification of the cDNA clone (9).

Based on the pattern of amino acids near the signal sequence cleavage site (18), we assigned the first 17 amino acids as the signal peptide and localized the signal peptide cleavage site between Ser (amino acid 17) and Leu (amino acid 18). However, the NH₂-terminal sequence of the mature protein purified from A549 cells was Ala (amino acid 37). It is possible that following cleavage of the signal peptide, the protein is further processed to a mature form by proteolytic cleavage of 19 amino acids from the NH₂ terminus. Similar processing has been reported for generation of the mature form of IL-8 (13).
The genomic structure of ENA-78 consists of four exons and three introns showing great similarity to other inflammatory cytokines including IL-8, GRO-α, β, γ, platelet factor 4, and β-thromboglobulin. The ENA-78 gene was mapped to chromosome 4q13-21, clustering at the same locus as other inflammatory cytokines of the CXC family. These two observations suggest that ENA-78 evolved together with other members of the CXC family of neutrophil activating peptides from a primordial gene by gene duplication.

The production of interleukin-8 and ENA-78 was not constitutive, but rather was induced by interleukin-1, TNF-α, or PMA (2, 9). The induction of IL-8 gene expression has been shown to occur at the transcriptional level. The 5'-flanking region of IL-8 contains two cis elements, one is the NF-κB-binding site, the other is the C/EBP element. In the promoter of the IL-8 gene, either element alone was not sufficient for induction by IL-1, TNF-α, or PMA suggesting that both elements are required and cooperative for the induction of IL-8 transcription by the three stimuli (14).

The 5'-flanking region of the ENA-78 gene also contained putative NF-κB and C/EBP regulatory elements. However, in contrast to the IL-8 gene, the NF-κB element alone in the ENA-78 gene was sufficient for the response to the induction, and there was no synergism between these two elements in the 293 cells. The IL-6 gene also contains both NF-κB and NF-IL-6 (similar to C/EBP) elements. However, some investigators reported that NF-κB alone may suffice for the induction of IL-6 transcription by IL-1 or TNF (24), while others suggested that the NF-IL-6 (C/EBP) element is important, or that multiple regulatory elements may be involved (25-27). The contradictory data may be due to differences in the transfection systems used for gene expression. The structure of the 5'-flanking region of the IL-5 gene is different from that of the IL-6 gene in that the NF-κB-binding site is located 70 bp downstream of the NF-κB-binding site in the IL-6 gene, while both elements are located adjacent to each other (only 3 bp apart) in the IL-8 gene. This difference may explain why both elements were required in the IL-8 gene. In this regard, the NF-κB element in the ENA-78 gene is 16 bp downstream from the putative C/EBP element. The separation of these two cis elements, as in the case of the IL-6 gene, may account for the independence of the NF-κB element in the induction of the ENA-78 gene.

In addition, the sequence of the NF-κB element in ENA-78 is GG-GAATTTC, while the sequence of the NF-κB element in the IL-8 gene is GGAATTTCC and in the IL-6 gene is GGAATTTCC. NF-κB elements of the ENA-78 and IL-6 genes have three consecutive Gs. It has been shown that the three consecutive Gs in the 5' half-site of NF-κB sequences is important for the binding of NF-κB to DNA (28-30). Single base substitution in the 5'-half-site of the NF-κB element can result in changes in the binding of different subunits of transcription factor. Therefore, NF-κB element in the regulatory region of immune response genes differing only by a single nucleotide can result in profound alterations in NF-κB/Rel subunit binding characteristics. This difference in subunit binding characteristics may account for the independence of the NF-κB element of the ENA-78 gene in its regulation.

It has been shown that the IL-8 NF-κB element is unable to bind to NFκB1 homodimers and NFκB1-RelA heterodimer; it only binds to the RelA, NFκB2, and C-Rel homodimers, suggesting that prototypical NF-κB is not involved in NF-κB regul
lates expression of the IL-8 gene and there is selective involvement of RelA in IL-8 gene expression. Since the ENA-78 and IL-8 genes showed different mechanisms in the interaction of regulatory elements of C/EBP and NF-κB, it is possible that different subunits of NF-κB transcription factor are involved in the gene regulation of the ENA-78 gene.

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