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ENHANCER FUNCTION OF A 53-BP REPETITIVE ELEMENT IN THE 5' FLANKING REGION OF THE HUMAN NEUTROPHIL ELASTASE GENE

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SUMMARY: Expression of the human neutrophil elastase (NE) gene is limited to the early stage of myeloid cell differentiation in bone marrow cells. While NE gene expression is controlled mainly at the transcriptional level during bone marrow cell differentiation, the mechanism of transcriptional control is not fully understood. One motif of interest in the 5' flanking region of the gene is the six tandem repeats of a 53-bp nucleotide sequence (REP53) containing a potential binding site for a basic helix-loop-helix protein located at -1032 to -716. The REP53 sequence can function as a non-cell specific transcriptional enhancer which is capable of augmenting heterologous promoter activity. When the single REP53 element was inserted into the pAZ1037 chloramphenicol acetyltransferase (CAT) expression vector immediately upstream of the chicken β-actin promoter in either normal or inverted orientation and used to transfect K-562 erythroleukemia or HeLa cervical carcinoma cells, these modified vectors achieved 2 to 3-fold higher CAT activity than the parental pAZ1037 vector irrespective of orientation of the REP53.

Human neutrophil elastase (NE ; EC 3,4,21,37) is a potent serine protease stored in the primary (azurophilic) granules of mature neutrophils (1-5). NE plays an essential role in the pathogenesis of pulmonary emphysema through its enzymatic activity to destroy the alveolar architecture of the lung (6). The NE protein is coded by a single copy gene composed of 5 exons and 4 introns (7,8). Although mature neutrophils carry large amounts of NE and release the enzyme when neutrophils are activated or disintegrated (2,4,6), the NE gene is not expressed in mature neutrophils (9,10). Namely, expression of the NE gene is limited to the early stage of myeloid cell differentiation in bone marrow cells, mostly in promyelocytes (9,10). NE

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ABBREVIATIONS:
NE: Neutrophil Elastase, CAT: Chloramphenicol Acetyltransferase, bHLH: basic helix-loop-helix.

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gene expression is controlled mainly at the transcriptional level during bone marrow cell differentiation towards either the myelocytic or mononuclear phagocytic linage (11). While the 5' flanking region of the NE gene contains essential promoter elements such as TATA and CAAT boxes like other regulated genes, the mechanism of tightly cntrolled transcriptional modulation of the NE gene is not well understood (7,8,11). One motif of interest is the six tandem repeats of a 53-bp nucleotide sequence (REP53) starting at the position of -1032 to -716 relative to the transcription initiation site (7,8). In the present report, we describe enhancer function of the REP53 that can activate a heterologous promoter in cells which normally do not express the NE gene.

METHODS

Cell Culture

The K-562 erythroleukemia cell line [American Type Culture Collection (ATCC) CCL243] and HeLa cervical carcinoma cell line (ATCC CCL2) were maintained at 37°C in Dulbecco's modified Eagle's medium (Whittaker Bioproducts) suppremented with 10% fetal bovine serum, 4 mM glutamine, 50 units/ml penicilline and 50 µg/ml streptomycin (all from Biofluids). K-562 cells were used when cells were in exponential growth (5-10 x 10^5/ml); HeLa cells were used when cells were 80-90% confluent.

Preparation of REP53 Element

The 53-bp repetitive element in the 5' flanking region of the NE gene (REP53) (references 7,8; Figure 1) was synthesized by oligonucleotide synthesizer (Applied Biosystems). Briefly, two complementary strands of REP53 containing artificial *Bam*HI or *Bgl*II restriction site at each end (for the sense strand, 5'-*Bam*HI, 3'-*Bgl*II; for the antisense strand, 5'-*Bgl*II, 3'-*Bam*HI, respectively) were synthesized, annealed to generate the double-stranded REP53, and purified using gel electrophoresis.

Evaluation of Function of REP53 with a Heterologous Promoter using Chloramphenicol Acetyltransferase Assay in K-562 and HeLa Cells

To evaluate the function of the REP53 motif, a single REP53 element was inserted into the chloramphenicol acetyltransferase (CAT) expression plasmid vector pAZ1037 (12). The double-stranded REP53 was prepared as described above. The pAZ1037 plasmid was cleaved at the unique *Xho*I site immediately 5' to the chicken β-actin promoter, the *Xho*I end blunted by T4 DNA polymerase (New England Biolabs), *Bgl*II linker (Pharmacia-LKB) ligated and finally digested with *Bgl*II to make cohesive ends. The REP53 element was then inserted into the vector in either normal or inverted orientation to generate the modified plasmid pREP53s or pREP53a, respectively (Figure 2A). The insert was confirmed by direct sequencing using the Sanger method (13).

These CAT plasmid vectors pAZ1037, pREP53s and pREP53a (10 µg/each) were used to transfect K-562 and HeLa cells, which are normally not expressing the NE gene (9,11). As a negative control, pSV0cat, a CAT plasmid vector containing no promoter sequence was used (14). K-562 cells (10^7 cells) were transfected by electroporation (2000 V, 25 µF; Gene Pulser,

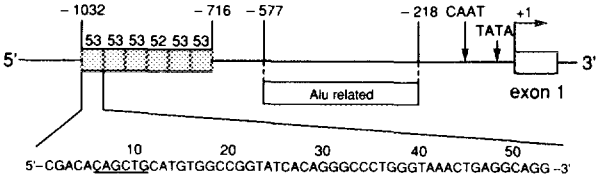

Figure 1. The 53-bp repetitive element in the 5'-flanking sequence of the NE gene. *Top* - structure of the NE 5' flanking region. *Bottom* - nucleotide sequence the 53-bp repeat (REP53). The nucleotide sequence homologous to the E-box consensus motif is shown underlined.

Bio-Rad) (15), and HeLa cells (5 x 10^6 cells/plate) were transfected with cationic liposomes (Lipofectin, BRL) (16). To normalize CAT activity in cells transfected with different CAT vectors, cells were co-transfected with 5 μg of the firefly luciferase expression vector pRSVL (17). After 48 h, CAT activity along with luciferase activity was evaluated. K-562 cells were washed twice with phosphate bufferd saline, pH 7.4 (PBS, Mediatech). HeLa cells were first detached with trypsin/EDTA (Biofluids) and scraped, and then washed with PBS. These cells were then resuspended in lysis buffer [100 mM pottasium phosphate, pH 7.8, 1 mM dithiothreitol (Sigma)], followed by 3 cycles of freezing and thawing, centrifugated (16,000 x g) at 4°C for 5 min, and the cell lysate supernate was recovered (17). CAT activity was assayed by the standard method using [^{14}C]chloramphenicol (Amersham) as described by Gorman et al. (14) and quantified by phosphoimaging of thin layer chromatography sheets. Luciferase activity was analyzed as described by de Wet et al. (17) using a Monolight 2010 luminometer (Analytical Luminescence Laboratories). The protein concentration of cell lysate was measured by the Bradford method (Bio-Rad Protein Assay, Bio-Rad) (18). Levels of CAT activity were normalized by luciferase activity, and were expressed as the average of relative CAT activity compared to that of the parental pAZ1037 after subtracting background CAT activity with pSV0cat.

RESULTS

Enhancement of Function of the Heterologous Promoter by Neutrophil Elastase Gene REP53

The REP53 sequence revealed enhancer function for the heterologous promoter. In this regard, K-562 or HeLa cells transfected with either pREP53s or pREP53a (Figure 2A) demonstrated 2 to 3-fold higher CAT activity compared to the cells transfected with the parental pAZ1037 vector (Figure 2B). Importantly, no significant difference was observed between CAT activity achieved by pREP53s and pREP53a.

DISCUSSION

Neutrophil elastase is a human serine protease with potent proteolytic activity (1-5). It is capable of destroying a broad range of substrates including the protein components of the connective tissue matrix of most organs, leading to a serious degenerative disorder such as pulmonary emphysema (1-6). Althouth NE is mainly carried and released by mature neutrophils, the NE gene is not expressed in these neutrophils (9,10). In this regards, expression of the NE gene is limited to the early stage of myeloid cell differentiation, mostly in promyelocytes (9,10), and it is modulated mainly at the transcriptional level (11). The 5' flanking region of the NE gene contains essential promoter elements including CAAT and TATA boxes like other regulated genes (7,8,19). However, the mechanism of tightly regulated transcriptional control of the NE gene expression in bone marrow cells is not understood. One of the difficulties lies in the fact that it is not possible to reproducibly transfect HL-60 cells, the model cell line for promyelocytes which is among few cell lines normally expressing the NE gene (11). Attempts to transfect HL-60 cells with plasmid vectors containing the NE gene 5' flanking sequences linked to the CAT reporter gene were unsuccessful despite trying a variety of available methods (unpublished observations).

Among the nucleotide motifs with potential importance for expression of the NE gene is the six tandem repeats of a 53-bp sequence (REP53) observed at the position of -1032 to -716 relative to the transcription initiation site of the NE gene (7,8). In the present study, we observed enhancer function of the REP53 sequence when linked to the heterologous promoter

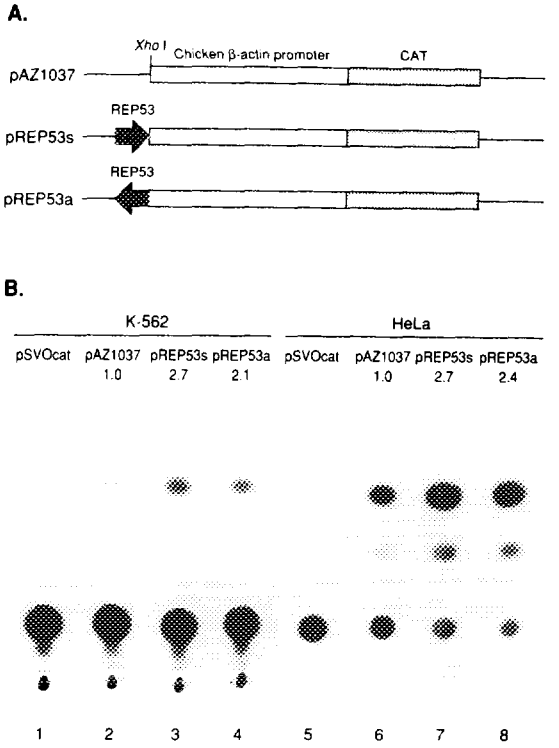

Figure 2. Enhancer function of the REP53 element. A. Schematics of plasmids pAZ1037, pREP53s and pREP53a. pREP53s and pREP53a were constructed from pAZ1037 by inserting a single REP53 element at the *Xho*I site in front of the chicken β-actin sequences [including the promoter, exon 1, intron 1 and partial exon 2 (12)] in either normal or inverted orientation, respectively. B. Examples of CAT assay data from K-562 erythroleukemia cells and HeLa cells transfected with 10 μg of either pSV0cat (negative control), pAZ1037, pREP53s, or pREP53a. K-562 cells were transfected by electroporation, and HeLa cells were transfected with cationic liposomes (Lipofectin, BRL). After 48 h, cells were recovered and CAT activity was quantified in cell lysates by phosphorimaging of thin layer chromatography sheets and normalized using luciferase expression directed by co-transfection of the pRSVL plasmid vector. Numbers above the columns indicate the average relative CAT activity compared to that of the parental pAZ1037 (K-562, n=4; HeLa, n=2). Lane 1 - K-562 cells transfected with pSV0cat. Lane 2 - same as lane 1, but with pAZ1037. Lane 3 - same as lane 1, but with pREP53s. Lane 4 - same as lane 1, but with pREP53a. Lane 5 - HeLa cells transfected with pSV0cat. Lane 6 - same as lane 5, but with pAZ1037. Lane 7 - same as lane 5, but with pREP53s. Lane 8 - same as lane 5, but with pREP53a.

(chicken β-actin in this case) in cells such as K-562 or HeLa, cells normally not expressing the NE gene (9,11). The role of REP53 in transcriptional regulation of the NE gene is unknown. However, in each 53-bp motif, there is one consensus E-box sequence (CAGCTG, +6 to +11 relative to the start of the repeat, Figure 1) that is known to be the recognition site for basic helix-loop-helix (bHLH) proteins (20,21). Transcription factors characterized by presence of a HLH domain, such as Myc, MyoD or myogenin, are known to play a central role in the regulation of cell growth/differentiation and tumorigenesis (20,22,23). Although the REP53 motif is not present in the 5' flanking region of other genes such as myeloperoxidase or cathepsin G, genes which expression is also limited to the early stage of myeloid differentiation (11), and Han et al. (24) have suggested that it does not modulate function of the NE gene promoter, at

least in U-937 histiocytic lymphoma cells, it is conceivable that the REP53 is playing some role in promyelocyte-specific expression of the NE gene.

The present study demonstrates that REP53 is capable of functioning as a non-cell specific transcriptional enhancer. In this regard, REP53 may be useful to facilitate expression of heterologous genes in bone marrow as well as non-hematopoietic cells.

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