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Egr Family Member Proteins Are Involved in the Activation of the Cathepsin L Gene in *v-src*-Transformed Cells

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Both the protein and mRNA levels of cathepsin L in SR-3Y1-2, *v-src*-transformed 3Y1 cell lines were higher than in 3Y1 cells. Results of CAT assays suggested that the v-src responsible region in the cathepsin L gene localizes to 300 bp in the 5′-upstream region and 60 bp in the first exon. DNaseI footprinting analyses showed that transcription factors bind to the region from 29bp to 55bp from the transcription start site. This region contains a CAAT-box and a 5′-GGCGGGGGCGG-3′ sequence containing two repeat copies of consensus Sp-1 binding sites, overlapping a consensus Egr family binding site. DNA band shift assays showed that Sp-1 and NF-1 binding proteins bind to this region in 3Y1 cells while Egr family protein binds in SR-3Y1-2 cells. These results suggest that Egr family proteins are involved in the activation of the cathepsin L gene in SR-3Y1-2 cells.

Cathepsin L is localized mainly in lysosomes and plays an important role in intracellular protein degradation (1). cDNA and gene cloning of cathepsin L showed its amino acid sequence to be related to those of cathepsins B and H, but that the gene structures are divergent in length and have different exon-intron structures (2,3). The cathepsin L gene contains Sp-1 binding sequences but no TATA-box in the 5′-upstream region. Hence, cathepsin L is considered to be a housekeeping gene (4).

The major excreted protein secreted from *v-ras*-transformed NIH3T3 cells has been identified as the precursor form of cathepsin L, procathepsin L (5,6). *v-ras* mediated transformation causes an increase in the level of the mRNA for cathepsin L but a decrease in the intracellular cathepsin L level (7). These facts suggest that signal transduction systems from the v-Ras protein affect not only the regulation of the cathepsin L gene but also the sorting mechanism of cathepsin L to lysosomes. Procathepsin L is also secreted from Sertoli cells, the endometrium, and placental trophoblast under physiological conditions (8-10). These secretions accompany the estrus cycle.

Cathepsin L gene transcription is also stimulated in NIH3T3 cells by treatment with PDGF and PMA. This induction requires *de novo* protein synthesis since it can be prevented by cyclohexamide (11). Thus, the cathepsin L gene is a so-called 'delayed early' gene whose transcription increases after the increase in 'immediate early' gene transcription and before DNA duplication (12). 'Delayed early' genes are the targets of 'immediate early' gene products such as c-Fos, c-Jun, and Egr family proteins. Egr family proteins, including Egr1-4 and WT1, are transcription factors bound to the nucleotide sequence 5′-GCGG/TGGGCG-3′(13). This consensus nucleotide sequence occurs in the cathepsin L 5′-upstream region. Egr-1 is, of particular interest since Egr-1 is induced by PMA, PDGF, cAMP, NGF, etc., which also cause the induction of cathepsin L mRNA.

In this paper, we demonstrate that the cathepsin L gene is activated in SR-3Y1-2 cells, which are 3Y1 cells transformed by *v-src* (14,15). A region in the cathepsin L 5′-upstream region with the nucleotide sequence 5′-CACAGCCAATGACGGGGCGGGGGCGG-3′ binds to Egr family proteins in SR-3Y1-2 cells while in 3Y1 cells Sp-1 and NF-1 are bound.

Abbreviations used: bp and kbp, base pair(s) and kilobase pair(s); BSA, bovine serum albumin; CAT, chloramphenycol acetyl transferase; CRE, cAMP response element; DMEM, Dulbecco's modified minimum essential medium; DTT, dithiothreitol; FCS, fetal calf serum; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; kDa, kilodalton; PAGE, polyacrylamide gel electrophoresis; PBS (−), Dulbecco's phosphate buffered saline; PDGF, platelet derived growth factor; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; SDSa, sodium dodecyl sarcosinate.

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MATERIALS AND METHODS

Materials used in this study were obtained as follows: DNaseI, competitor oligonucleotides Sp-1 (5′-ATTCGATCGGGGCGGGGC-GAAGC-3′ and its complementary sequence) and NF-1 (5′-CCTTTG-GCAATGCTGCCAATATG-3′ and its complementary sequence) from Promega, and poly d[I-C] from Boehringer Mannheim. Other materials has been described elsewhere (2,16).

Two synthetic oligonucleotides, Zif (5′-GATCTCGCGGGGGCG-AGGGGGATC-3′ and its complementary sequence), a competitor for the Egr family protein binding site but not for the Sp-1 binding site, and MUT (5′-GATCTCGCGGGTGCGAGGGGGATC-3′ and its complementary sequence), a competitor for neither the Egr family protein nor Sp-1 binding sites (17), and PCR primers to amplify the 5′-upstream region (see below) were synthesized using an Applied Biosystem 381A DNA synthesizer.

The rat fibroblast cell line 3Y1 and its *v-src* mediated transformant cell line SR-3Y1-2 were cultured in DMEM supplemented with 10% FCS and penicillin G (150U/ml), streptomycin (150U/ml), and amphotericin B (375ng/ml) under 5% CO_2 in a humidified gas incubator at 37°C.

Total cell extracts for SDS-PAGE and immunoblot analyses were prepared as follows. Cells under subconfluent conditions were washed twice with PBS (−) and harvested with a rubber policeman. The cells were suspended in 500 μl PBS(−) and disrupted by sonication. The protein concentrations of the total cell extracts were determined with a BCA protein assay kit using BSA as a standard according to the manufacturer's protocol. SDS-PAGE in 12.5% gels (18) and immunoblotting (19) with anti-rat cathepsin L antibody were described previously (20). Antibody bound to antigens was combined with anti-rabbit antibody conjugated to horseradish-peroxidase and detected with a Konica immunostaining HRP kit.

All techniques for the manipulation of DNA and RNA were carried out according to established protocols (21) unless specified otherwise. RNA blot hybridization analyses with a [^{32}P]-labeled cDNA probe for rat cathepsin L were performed as described previously (2). The amounts of hybridized probes were determined by counting the bands with an Image Analyzer BAS 2000 system.

Nuclear extracts from 3Y1 and SR-3Y1-2 cells were prepared according to Schreiber *et al.* (22), and the concentrations were determined from OD_{280}/OD_{260} (23). Probes for DNaseI footprinting and DNA band shift assays were generated by PCR with [^{32}P]-labeled primer (5′-GATCCCCGGAGGTCGCCGAGG-3′) and reverse primer (5′-CGGGTTATACAAACCTCCACCCTG-3′) using the plasmid bearing rat cathepsin L 5′-upstream region as a template. A DNA fragment about 360bp in length containing the 5′-upstream region and a part of exon 1 including the transcription start site but not the coding region of the rat cathepsin L gene was purified by polyacrylamide gel electrophoresis. The electrophoretic markers were generated from probes by purine specific cleavage (A+G) according to the method of Maxam-Gilbert (24).

DNaseI footprinting was carried out as described (25). Reaction mixtures containing 1×10^4 cpm probe (approximately 3 fmol), 50μg of nuclear extract or BSA as a control, 25mM HEPES-KOH (pH7.9 at 4°C), 80mM NaCl, 0.5mM EDTA, 10% glycerol, 1mM DTT, and 3mM PMSF in the presence or absence of competitor oligonucleotide (approximately 1.7pmol) were incubated at room temperature for 30min after which 0.4 units of DNaseI (diluted in 5mM $CaCl_2$, 10mM $MgCl_2$) was added. The reactions were stopped after 45 seconds by the addition of 100μl of 100mM Tris-HCl (pH8.0), 100mM NaCl, 1% SDSa, 10mM EDTA, and 25μg/ml denatured salmon sperm DNA. The samples were subjected to two cycles of extraction with phenol/$CHCl_3$ and ethanol precipitation. After drying, the samples were fractionated on buffered gradient denatured 6% polyacrylamide gels containing 8.3M urea (26). The gels were fixed with 10% acetate and 10% methanol, dried, and subjected to autoradiography.

DNA band shift assays were carried out as follows (27). Reaction mixtures (10μl) containing 1×10^4cpm of probe (approximately 3 fmol), 2.5μg of nuclear extract, 2μg of poly d[I-C], 25mM HEPES-KOH (pH7.9 at 4°C), 50mM KCl, 0.5mM EDTA, 10% glycerol, 1mM DTT, and 3mM PMSF in the presence or absence of competitor oligonucleotide (1.7 pmol) were incubated at room temperature for 30 min and subjected to electrophoresis in 3.6% polyacrylamide gels (30/1) in the TGE gel system [running buffer consisting of 50mM Tris, 250mM glycine, 2mM EDTA, pH8.5; gels containing the same buffer as the running buffer plus 3.4% glycerol (W/V)]. The gels were fixed with 10% acetate and 10% methanol and subjected to autoradiography.

RESULTS AND DISCUSSION

The v-*ras* mediated transformation of NIH3T3 cells causes vast amounts of procathepsin L secretion due

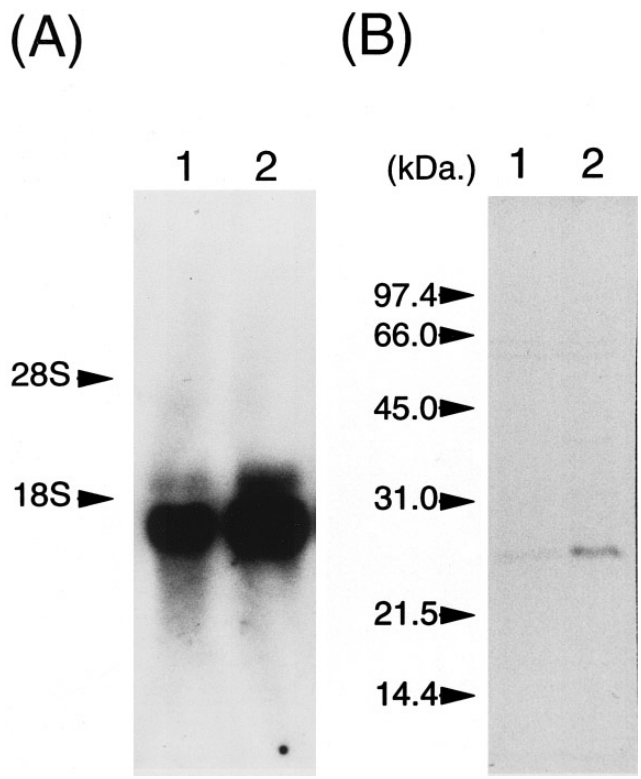

FIG. 1. *RNA blot hybridization (A) and immunoblot (B) analyses of cathepsin L in 3Y1 and SR-3Y1-2 cells.* (A) Ten micrograms of total RNA extracted from 3Y1 (lane 1) and SR-3Y1-2 (lane 2) cells was fractionated in denaturing 1% agarose gels containing 2.2M formaldehyde and transferred onto nylon membranes. After prehybridization, the filters were hybridized with [^{32}P]-labeled rat cathepsin L cDNA as a probe. Labeled cDNA probe hybridizing with the mRNA for cathepsin L was detected by autoradiography. The arrowheads on the left indicate marker ribosomal RNAs, with 28S migrating as 4.5kb and 18S as 1.9kb. (B) Fifty-micrograms of total cellular protein from 3Y1 (lane 1) and SR-3Y1-2 (lane 2) cells was subjected to 12.5% SDS-PAGE and the proteins were transferred onto PVDF membranes. Antibodies binding to antigens on the membranes were combined with peroxidase-conjugated anti rabbit antibody and visualized with Konika immuno-staining kits according to the manufacturer's protocol. Numbers on left indicate the molecular weight markers: phosphorylase b, 97.4kDa; BSA, 66kDa; ovalbumin, 46kDa; carbonic anhydrase, 31kDa; trypsin inhibitor, 21.5kDa; and lysozyme, 14.4kDa.

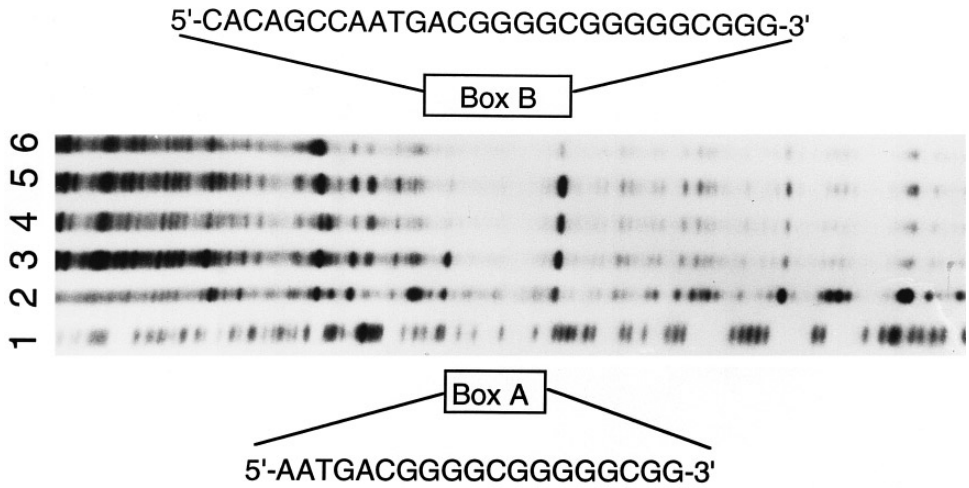

FIG. 2. *DNaseI footprinting analyses of the 5′-upstream region of the cathepsin L gene.* Ten thousand cpm of probe (see text) was incubated with 50 μg of BSA (lane 2) or nuclear extract from 3Y1 cells (lanes 3-5), SR-3Y1-2 cells (lane 6) in the presence or absence of oligonucleotide competitors. Lanes 3 and 6 contained no competitor, lane 4 contained Sp-1 oligonucleotide, and lane 5 contained NF-1 oligonucleotide. Lane 1 shows the sequence markers generated from the probe by purine-specific cleavage (A+G). Box-A on the left and box-B on the right indicate the nuclear extract protection sites in 3Y1 and SR-3Y1-2 cells, respectively.

to in part an increase in the level of its mRNA (6). To investigate whether the cathepsin L gene is activated by transformation with another oncogene, *v-src*, we analyzed the mRNA for cathepsin L in both SR-3Y1-2 cells and parent 3Y1 cells by RNA blot hybridization analysis. Figure 1A shows that the mRNA for cathepsin L in both cells migrate as a 1.7kb band. The amount of cathepsin L mRNA in SR-3Y1 cells was about 3-fold greater than in 3Y1 cells.

Next, intracellular cathepsin L protein levels were analyzed by immunoblot analysis with anti-rat cathepsin L antibody (Fig. 1B). Three species of protein were detected in SR-3Y1-2 cells: Procathepsin L with a molecular mass of 39 kDa., the single chain form of cathepsin L with a molecular mass of 31 kDa., and the heavy chain of two chain form of the mature enzyme with a molecular mass of 24 kDa. In contrast, only the heavy chain of two chain form is present in 3Y1 cells. The amount of intracellular cathepsin L in SR-3Y1-2 cells was about 5-fold greater than in 3Y1 cells. Only a faint band corresponding to secreted procathepsin L was detected in the medium from SR-3Y1-2 cells while no band was detectable in the medium from 3Y1 cells (data not shown).

NIH3T3 cells transformed by *v-ras* show an increase in the levels of secreted procathepsin L while the levels of cellular cathepsin L decrease in comparison with the parent cells (7). Thus, the *v-ras* gene affects not only the regulation of the cathepsin L gene but also the sorting of cathepsin L to lysosomes, while the v-*src* gene only activates the cathepsin L gene.

The 5′-upstream region of the rat cathepsin L gene contains two AP-2 binding sites, one CRE, one CAAT box, and four Sp-1 binding sites, two of which are sequentially repeated. This region also includes an Egr family binding consensus nucleotide sequence, 5′-GCGGGGGCG-3′ (3, 27). The results of CAT assays after transfection with CAT reporter plasmid bearing 300bp of the 5′-upstream region and 60bp of the first exon of the rat cathepsin L gene show CAT activities in SR-3Y1-2 cells to be 14-fold greater than in 3Y1 cells: 2.9% acetylation was observed in 50μg of cellular extract from transfected 3Y1 cells after 30 min, while 41.5% acetylation occurred in SR-3Y1-2 cells. These extracts contained the same β-galactosidase activities derived from the control pCH110 plasmids bearing *lacZ*. This region, therefore, is presumably involved in the activation of the cathepsin L gene by *v-src*-mediated transformation.

At first, to identify the transacting factor binding region in the rat cathepsin L gene, DNA footprinting analyses with nuclear extracts from 3Y1 and SR-3Y1-2 cells were performed (Fig. 2). Nuclear proteins prepared from 3Y1 cells bound to the region of the cathepsin L gene with the nucleotide sequence 5′-AATGACGGGGCGGGGGCGG-3′ (see lane 3 and box A). The other region failed to be protected by DNaseI. When nuclear extracts from 3Y1 cells were used with competitor oligonucleotides containing either an Sp-1 or NF-1 binding site, the site hypersensitive to DNase I on the 5′-end of the protective region disappeared (lanes 4 and 5). Thus, nuclear proteins from 3Y1 cells includes both Sp-1 and NF-1 binding proteins which also bind to box A in the rat cathepsin L gene.

The nucleotide sequence of the region in the cathepsin L gene that binds nuclear proteins prepared from SR-3Y1-2 cells to the cathepsin L gene is 5′-CACAGCCAATGACGGGGCGGGGGCGGG-3′ (see lane 6 and

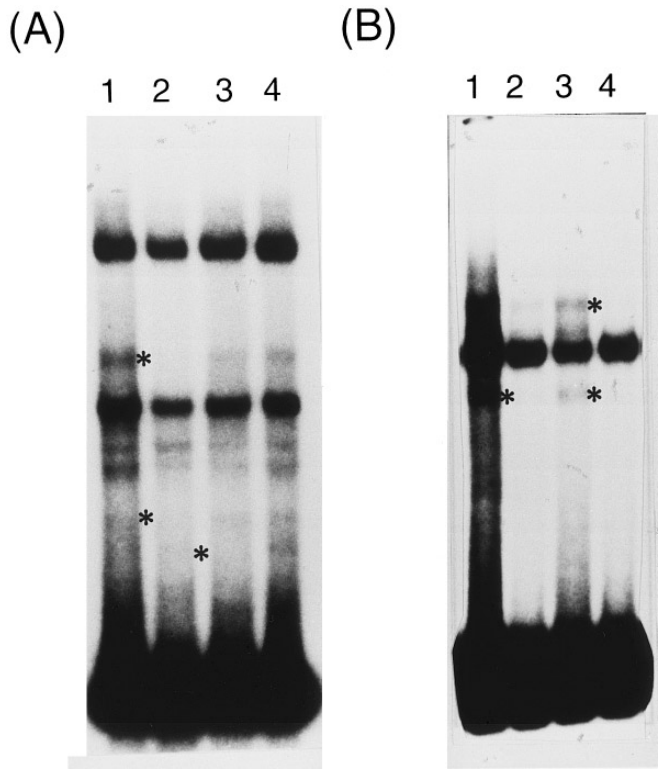

FIG. 3. *DNA band shift assays in the presence or absence of competitor oligonucleotide.* (A) Ten thousand cpm of probe (see text) was incubated with 2.5μg of nuclear extracts from 3Y1 cells in the absence or presence of 3 pmol of oligonucleotide competitors; lane 1, in the absence of competitor DNA; lane 2, in the presence of Sp-1 oligonucleotide; lane 3, in the presence of NF-1 oligonucleotide; lane 4, in the presence of Zif oligonucleotide competitor. Probes that bind no nuclear protein migrated to the bottom of the gel. Asterisks indicate the positions of shifted-bands that disappeared in the presence of competitor oligonucleotide. (B) The same conditions as (A) except that the nuclear extracts were prepared from SR-3Y1-2 cells; lane 1, no competitors; lane 2, Zif competitor oligonucleotide; lane 3, MUT competitor oligonucleotide; lane 4, Sp-1 competitor oligonucleotide. Asterisks show the positions of bands that disappeared in the presence of oligonucleotides.

box B). There is no other region that binds the nuclear extracts. In the case of the nuclear extracts from SR-3Y1-2 cells, Egr-1, an 'immediate early' gene product, is of particular interest because Egr-1 is a transcription factor that recognizes the nucleotide sequence 5′-GCG-GGGGCG-3′ and is induced in accordance with *v-src* mediated transformation (11, 28). The protective region include the consensus binding nucleotide sequence of the Egr family.

To resolve whether Egr family members bind to the cathepsin L gene, we performed DNA band shift assays with DNA in excess to nuclear proteins. Figure 3A shows that some of the shifted DNA probe can be observed when the nuclear extracts from 3Y1 cells are mixed with a [^{32}P]-labeled probe composed of 300bp of the 5′-upstream and 60 bp of the first exon. In the presence of oligonucleotide competitors for either the Sp-1 binding site or the NF-1 binding site, some of the shifted bands disappeared compared with the profile in the absence of oligonucleotide competitors. In contrast, the Zif oligonucleotide competitor produced no change in the profile of the shifted DNA bands. Thus, both Sp-1 and NF-1, but not Egr family members bind to the cathepsin L gene in 3Y1 cells. In the case of the nuclear extracts from SR-3Y1-2 cells, some shifted DNA bands were observed (Fig. 3B). In the presence of either the Zif oligonucleotide competitor or the Sp-1 oligonucleotide competitor, two of the shifted bands were not observed, while in the presence of the MUT oligonucleotide competitor, the same shifted DNA band profile was observed as in absence of competitor oligonucleotides. Thus, in the nuclear extracts from SR-3Y1-2 cells, both Sp-1 binding proteins and Egr family proteins bind to the cathepsin L gene. Further, we tried super-shift analyses using anti-Egr-1 antibody, but failed to demonstrate Egr-1 binding to this region of the cathepsin L gene. Other reports of failed supershift analyses using the anti-Egr-1 antibody suggest that the available antibody is unsuitable for supershift analyses (29, 30).

The cathepsin L gene is activated not only by *v-ras* transformation, but also by treatment with PDGF and PMA (6). One candidate transcription factor in this induction is the AP-1 complex composed of a heterodimer of c-Fos and c-Jun (31), but this is not true for the induction of the mRNA for cathepsin L: There is no AP-1 complex binding site in the 5′-upstream of cathepsin L gene. In addition, co-transfection of 3Y1 cells with expression vectors bearing the cDNAs for *c-fos* and *c-jun* fails to induce cathepsin L mRNA (unpublished data). Recently, an EJ/v-Ha-*ras* transformed NIH3T3 cell line has been shown to possess small amounts of the mRNA for cathepsin L even though this cell line has large amounts of the mRNA for *c-jun;* in contrast, NIH3T3 cells transformed by the *RAS1*^{Leu}del gene express large amounts of the mRNA for cathepsin L but have no c-Jun protein (32). Recently, it was reported that the switching mechanism for Sp-1 and Egr-1 binding to the same region in the PDGF-A gene is controlled in vascular endothelial cells. In the absence of PMA in the medium, Sp-1 binds to the PDGF-A gene, while in the presence of PMA, Egr-1 binds to the PDGF-A gene through the same region as Sp-1 binding and causes gene activation (33). This mechanism might also apply to the cathepsin L gene. A strain of egr-1 knock-out mice shows a loss of the estrous cycle (34). Secretion of procathepsin L from the endometrium and Sertoli cells possibly is lacking in this knock-out strain. These hypotheses require further proof for confirmation.

In this study, we show that both Sp-1 and NF-1 binding proteins in 3Y1 cells, in contrast to Egr family proteins, presumably Egr-1, in SR-3Y1-2 cells, bind to a 5′-upstream region of the cathepsin L gene localized

29 to 55 bp from the transcription start site and having the nucleotide sequence 5′-CACAGCCAATGACGG-GGCGGGGGCGGG-3′. To prove whether Egr family proteins participate in cathepsin L gene activation in v-src transformed cells further analyses using anti-sense oligonucleotides and specific antibodies against Egr family members are necessary.

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