Regulation of the gene promoter for extracellular signal-regulated protein kinase 2 by transcription factors NF-Y and Sp3

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We have previously shown that the maximal promoter activity of the gene for extracellular signal-regulated protein kinase 2 (ERK2; also known as p42 mitogen-activated protein kinase) resides in the 371 bp 5'-flanking sequence. In the present study we defined roles for a CCAAT box and two adjacent GC boxes in the activity of this promoter. Deletion analysis and DNase I footprinting of this 371 bp region indicated that the CCAAT box at -64 and GC boxes at -86 and -39 are crucial for promoter activity. Electrophoretic mobility-shift assays showed that transcription factor NF-Y/CBF binds to the CCAAT box. Sp1 and Sp3, members of the Sp family of transcription factors, bind to the GC boxes of the ERK2 promoter. The binding of Sp3 was

predominant over that of Sp1. Disruption by mutation of any of the CCAAT box and GC boxes similarly decreased promoter activity. These three *cis* elements exhibited a moderate synergy in promoter function. The transactivating role of NF-Y was corroborated by the finding that a dominant-negative form of NF-YA diminished the promoter activity. These results provide clues for refining our understanding of not only the regulation of expression of the gene for ERK2 but also mechanisms by which NF-Y and Sp1/Sp3 regulate transcription.

Key words: CCAAT box, gene expression, MAP kinase, transcription.

INTRODUCTION

The mitogen-activated protein kinase (MAP kinase) cascade mediates various extracellular signals through distinct sets of MAP kinase modules via an evolutionarily conserved mechanism of sequential activation of protein kinases [1,2]. Extracellular signal-regulated protein kinase 2 (ERK2, also known as p42 MAP kinase) is an integral component of the ERK module that responds to extracellular signals committing cells to proliferation or differentiation. Recent studies, which include the identification of scaffold proteins for the kinases, have underscored the importance of mutual stoichiometries of the MAP kinase cascade components in regulating the mechanism of signal transduction [2]. The relative concentrations of the interacting counterparts can determine the intracellular localization of the components, as well as the amplitude and specificity of the downstream signal. Although a substantial amount of the closely related isoforms ERK2 and ERK1 (p44 MAP kinase) are expressed in a wide variety of tissues, variations exist in their expression levels [3,4]. Although functional redundancy of the ERK isoforms could be speculated from their similar biochemical properties, the unique expression patterns and the preference of the particular scaffold protein for a specific ERK isoform suggest that they might have unique roles in the MAP kinase cascade. Specific and differential regulation of the genes encoding the two isoforms would be an important indicator of mechanisms that confer each one's specificity of signalling through the MAP kinase cascade.

In elucidating the mechanisms that endow potent and regulated expression of the ERKs, detailed characterization of the promoter has been desirable. To address the issue, Pagès et al. [5] and we [6] have isolated the mouse genes for ERK1 and ERK2 respectively and identified the corresponding promoter sequences. Previous analysis of the ERK2 promoter revealed that the maximal transcription is driven by the 371 bp sequence immediately upstream of the translation start site [6]. In the present study we elucidated the importance of the CCAAT box and GC

boxes in this 371 bp region and showed that transcription factors NF-Y (also called CBF) and Sp3 are responsible for regulating these *cis*-acting elements in the transcriptional control of the ERK2 promoter.

EXPERIMENTAL

Preparation of nuclear extracts and DNase I footprinting analysis

Mouse P19 EC (embryonal carcinoma) cells were maintained as described previously [6]. Nuclear extracts were prepared as described [7] from differentiated P19 cells that had been treated with 1 μ M retinoic acid for 3 days.

Footprinting was performed as follows. Restriction fragments were end-labelled with [γ -³²P]ATP. Approx. 7 fmol of probe was incubated on ice with nuclear extract for 60 min in 50 μ l of 20 mM Hepes/KOH (pH 7.9)/6.25 mM MgCl₂/0.5 mM EDTA/50 mM KCl/0.5 mM dithiothreitol/10% (v/v) glycerol in the presence of 1 μ g of poly(dI-dC) (Amersham Pharmacia Biotech). After the addition of 37.5–200 ng of DNase I (grade I; Boehringer Mannheim) in 3 μ l of 33.3 mM CaCl₂, digestion proceeded on ice for 90 s. The reaction was stopped by the addition of 100 μ l of 0.2 M NaCl/20 mM EDTA/1% (w/v) SDS/250 μ g/ml tRNA/100 μ g/ml proteinase K. After incubation at 37 °C for 30 min, samples were extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1, by vol.), precipitated with ethanol and subjected to electrophoresis on a 5% (w/v) polyacrylamide/7 M urea gel.

Electrophoretic mobility-shift assay (EMSA)

Gel-purified double-stranded oligonucleotides were labelled with [γ -³²P]ATP by using T4 polynucleotide kinase. Unincorporated radiolabel was removed with NICK columns (Amersham Pharmacia Biotech). Approx. 4.3 fmol of the labelled probe was

Abbreviations used: CAT, chloramphenicol acetyltransferase; C/EBP, CCAAT-enhancer-binding protein; CHO, Chinese hamster ovary; EMSA, electrophoretic mobility-shift assay; ERK, extracellular signal-regulated protein kinase; MAP kinase, mitogen-activated protein kinase.

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incubated with nuclear extracts in a 25 μ l reaction whose composition was the same as that for the footprinting experiment. Unless specified otherwise, 5 μ g of the differentiated P19 cell nuclear extract and 1 μ g of poly(dI-dC) were included in the reaction. The reaction was incubated for 30 min on ice and subjected to electrophoresis on a 4% polyacrylamide gel (acrylamide-to-bisacrylamide ratio 80:1; 1.5 mm thickness) in 0.25 $\times$ Tris/borate/EDTA buffer at 15 mA for 80–110 min at 4 °C. The sequences of double-stranded oligonucleotides used as competitor were as follows (only coding strands are shown): Sp1, 5'-GATCGATCGGGGCGGGGCGATC-3' [8]; NF-Y (E₂Y box), 5'-ATTTTCTGATTGGTTAAAGTTG-3' [9]; CCAAT-enhancer-binding protein (C/EBP), 5'-TGCAGATTGCGCAATCTGCA-3' [10]; NF-I, 5'-ATTTTGGCTTGAAGCCAA-TATG-3' [11]; CP2, 5'-GAGCAAGCACAACAGCCAA-TGAG-3' [12]. In the supershift experiments, nuclear extracts were incubated on ice with antibodies for 30 min before addition of the labelled probe. Polyclonal rabbit antibody against NF-YA N-terminus peptide was purchased from Rockland (Gilbertsville, PA, U.S.A.). Polyclonal rabbit antibody against NF-YB [13] was a gift from Dr R. Mantovani (University of Milano, Milano, Italy). Polyclonal rabbit anti-(CBF-C) (NF-YC) antibody raised against recombinant glutathione S-transferase-human CBF-C fusion protein [14] was kindly provided by Dr T. Taira (Hokkaido University, Sapporo, Japan). Anti-Sp1 (no. sc-59 X) and anti-Sp3 (no. sc-644 X) antibodies were purchased from Santa Cruz Biotechnology.

Chloramphenicol acetyltransferase (CAT) reporter plasmids

The construction of reporter plasmids described below was facilitated by the use of three unique restriction sites in pBS-fl0.57 [6], the *Aat*II site at -148 and the *Nae*I site at +98, both in the promoter sequence, and the *Sal*I site immediately downstream of the promoter sequence. Plasmids pCAT-B(-148/+223), pCAT-B(-42/+223) and pCAT-B(+99/+223) were described previously as pCAT-B(-148), pCAT-B(-42) and pCAT-B(+99) respectively [6]. Plasmid pCAT-B(-148/+98) was made from pCAT-B(-148/+223) by removal of the *Nae*I/*Sal*I fragment, followed by blunting and self-ligation. To generate mutant promoter CAT constructs with mutations in the CCAAT box and GC boxes, these sequences in plasmid pBS-fl0.57 were altered by using PCR-mediated mutagenesis. The double-mutant constructs were generated by introducing the desired second mutation into the single-mutant plasmid. The *Aat*II/*Sal*I fragment and *Aat*II/*Nae*I fragment of the mutated pBS-fl0.57 plasmids were subcloned into pCAT-Basic to generate pCAT(-148/+223) and pCAT(-148/+98)-derived mutant constructs respectively.

Cell transfection and reporter assays

Transient transfections and reporter gene assays with P19 EC cells were performed as described previously [6]. The CAT activity was assayed with the use of a phase-extraction method [15].

The co-transfection assay examining the effect of dominant-negative NF-YA was performed in Chinese hamster ovary (CHO) cells. The eukaryotic expression plasmids harbouring wild-type NF-YA cDNA (Δ 4YA1) or a dominant-negative NF-YA mutant (Δ 4YAm29 [16]) were gifts from Dr R. Mantovani. In brief, 1.5×10^5 cells were seeded on six-well plates 36 h before transfection. The cells were transfected with 150 ng of CAT construct and 10 or 50 ng of either Δ 4YA1 or Δ 4YAm29 plasmid by using 3 μ l of FuGENE 6 transfection reagent (Boehringer Mannheim). To adjust the total amount of DNA to 300 ng, pBluescript

SK(-) supplemented the transfected plasmids. Because promoter competition between the NF-YA expression plasmid and the normalization plasmid pCH110 (Amersham Pharmacia Biotech), which share the simian virus 40 promoter, was evident in the preliminary experiment, pCH110 was not included in this co-transfection. The cells were harvested 24 h after transfection and assayed for CAT activity.

RESULTS

Identification of positive *cis*-acting sequences in the ERK2 promoter

We have previously determined the structure of the mouse ERK2 gene and its promoter sequence [6]. In the present study we sought to define *cis*-acting sequences and transcription factors that regulate transcription of the mouse gene for ERK2. Previous CAT reporter assays with a series of 5'-deletion constructs showed that the maximal promoter activity is conferred by a 371 bp region immediately upstream of the translation start site [6]. To delineate further the region that is important to the promoter activity, we examined the promoter activity of four reporter constructs. The construct pCAT-B(-148/+98), which was generated by removing the downstream 125 bp from pCAT-B(-148/+223), showed promoter activity that was 50% of that of pCAT-B(-148/+223) but was still strong in differentiated P19 cells (Table 1). In contrast, the downstream regions in pCAT-B(-42/+223) and pCAT-B(+99/+223), which retained and lacked the transcription start sites respectively, had almost no activity. These results suggest that the essential promoter element(s) reside between nt -148 and -43. Although the sequence downstream of -42 does not exhibit a promoter activity on its own, it seems to be required for maximal transcription.

To delineate the *cis*-acting elements by their interaction with nuclear proteins, a DNase I footprinting assay was performed with a probe encompassing -174 to +100. As seen in Figure 1, nuclear extracts prepared from differentiated P19 cells revealed three regions that were protected from digestion with DNase I, indicating these sequences as potential *cis*-elements. Their locations corresponded to the CCAAT box at -64 and two GC boxes at -86 and -39 (Figure 2B). Hereafter we refer to the proximal GC box at -39 and the distal one at -86 as GC(I) and GC(II) respectively.

CCAAT box binds NF-Y, whereas GC boxes predominantly bind Sp3

To identify transcription factors that bound to the sequences that were protected in the footprinting assay, two double-stranded oligonucleotides spanning nt -93 to -51 and -72 to -27 were designed for EMSA such that each contained the potential *cis*-acting sequences, namely the CCAAT box and either of the two GC boxes, to examine nuclear protein binding to GC(I) and GC(II) separately; the -93/-51 probe contained GC(II) and the -72/-27 probe contained GC(I). The labelled -93/-51 probe formed a major nuclear protein-DNA complex (complex B in Figure 2A) and a minor complex (complex A) in all nuclear extracts tested. Incubation of the -72/-27 probe with nuclear extracts resulted in three major protein-DNA complexes (complexes D, E and F in Figure 2A) and a minor complex, C. Complex F, with the lowest mobility, was barely detectable at the lowest concentration of the nuclear extracts, whereas it represented a significant portion of the total shifted probe at the highest concentration of nuclear extracts (Figure 2A, lane 6), suggesting that complex F was a multimeric complex

Table 1 Deletion analysis of the 371 bp region of the ERK2 promoter

The reporter constructs with the deleted fragments of the 371 bp region were transiently transfected in undifferentiated P19 EC cells. The cells were treated with retinoic acid for 3 days and assayed for CAT activity. The CAT activity is represented in arbitrary units normalized for the β -galactosidase activity derived from the co-transfected normalization plasmid pCH110. Results are means $\pm$ S.E.M. for *n* independent transfections.

CAT construct	CAT activity (arbitrary units)	<i>n</i>
pCAT-B(-148/+223)	5499 $\pm$ 799	12
pCAT-B(-148/+98)	3006 $\pm$ 378	7
pCAT-B(-42/+223)	114 $\pm$ 26	4
pCAT-B(+99/+223)	52 $\pm$ 11	4

Figure 1 DNase I footprinting of the -174/+100 region of the ERK2 promoter

The -174/+100 fragment of the ERK2 promoter was labelled on either the coding (A) or non-coding (B) strand. The labelled probe was partly digested with DNase I in either the absence or the presence of increasing amounts of nuclear extracts (7.5, 15, 30 and 60 μ g, from left to right). The protected regions and the positions of their boundaries are indicated by open bars with numbers at their ends. Asterisks indicate hypersensitive sites. Lane 1, Maxam-Gilbert G + A sequence reaction.

composed of two or more of the faster-migrating complexes. The addition of a 125-fold molar excess of unlabelled Sp1 consensus site abolished the major complexes B and E, but had no effect on complex D (results not shown). These findings implied that Sp1 and/or its isoforms bound to GC(I) and GC(II). Because complex D was not affected by competition with the Sp1-binding site, we surmised that a protein binding to the CCAAT box formed complex D. When the labelled probe and nuclear extracts were

incubated with competitors with binding sites for distinct classes of CCAAT box-binding proteins such as NF-Y, NF-I, C/EBP and CP2, only the NF-Y site (Y box of the MHC class II E α gene) and unlabelled same sequence oligonucleotide competed out complex D (results not shown). This result suggested that transcription factor NF-Y binds to the CCAAT box to give rise to complex D. Although the -93/-51 probe contained the CCAAT box and the surrounding sequences, it did not form a complex corresponding to complex D. By using oligonucleotide probes with increasing 3' extensions flanking the CCAAT box, we found that the 8 bp 3'-flanking sequence of the -93/-51 probe was not sufficient to bind NF-Y (N. Sugiura and K. Takishima, unpublished work).

With these initial indications of what transcription factors might be responsible for binding to the CCAAT box and GC boxes, we performed a supershift analysis with antibodies against Sp1, Sp3 and NF-YA (the A subunit of the heterotrimeric NF-Y complex) either alone or in combination (Figure 2C). Preincubation of nuclear extract with anti-(NF-YA) antibody before the binding reaction resulted in the formation of a novel complex with the concomitant disappearance of complex D (Figure 2C, lanes 7, 10 and 11). In addition, complex D was supershifted with antibodies against other two NF-Y subunits, NF-YB and NF-YC (results not shown); we therefore confirmed the identity of the protein component in complex D as NF-Y. As seen in Figure 2(C), complex B obtained with the -93/-51 probe split into three bands (complexes B1, B2 and B3) after extended electrophoresis that gave better resolution. The intensity of band B1 was greater than those of complexes B2 and B3. Complex E, obtained with the -72/-27 probe, was actually composed of the intense complex E1 and faint E2. The minor complexes A and C each contained two species (A1 and A2, C1 and C2). The addition of anti-Sp1 antibody abolished complex B2; two supershifted species appeared instead (Figure 2C, lane 3). Anti-Sp3 antibody retarded the complex B1, generating two supershifted species (Figure 2C, lane 4) that were indistinguishable from those of the anti-Sp1-induced supershifted species derived from the complex B2 (compare lanes 3 and 4). Anti-Sp3 antibody also abrogated complex A2 (Figure 2C, lane 4). These two anti-Sp3-reactive species, complexes B1 and A2, seem to correspond to full-length and N-terminally truncated Sp3 isoforms [17] respectively. The assignment of two closely migrating complexes, the faster-migrating species B1 containing Sp3 and the slower B2 containing Sp1, is also consistent with previous studies [17-19]. In contrast, complex B3 was not affected by anti-Sp1 and anti-Sp3 antibodies (Figure 2C, lanes 3-5) and therefore might have contained another isoform of the Sp family such as Sp4 [20]. Similarly, as with the complexes formed with the -72/-27 probe, it was shown that complexes E1 and C2 contained the large and small Sp3 species (Figure 2C, lane 9) respectively and that the fainter complex E2 was derived from Sp1 (lane 8).

The identity of the transcription factors binding to the -93/-51 and -72/-27 probes was corroborated by introducing mutations into the CCAAT box and GC boxes of these EMSA probes. The CCAAT and GGGCGG sequences were altered to CCAAA [21] and GttCGG [22] respectively. The wild-type and mutant oligonucleotide probes were radiolabelled individually and employed in EMSA (Figure 2D). As expected, disruption of the GC box eliminated the formation of complexes B1, B2 and B3 (Figure 2D, lane 5) as well as complexes E1 and E2 (lane 2) but did not decrease NF-Y binding (lane 2). In contrast, the CCAAT box mutation abolished NF-Y binding, whereas an increase in the complexes bound to GC(I) was observed (Figure 2D, compare lanes 1 and 3). Similarly, the level of complex formation of NF-Y with the -72/-27 GC(I) mutant

Figure 2 NF-Y binds to the CCAAT box, whereas Sp3/Sp1 bind to the GC boxes

(A) EMSA showing nuclear protein binding to the -93/-51 probe and -72/-27 probe. Increasing amounts of nuclear extracts (2.5, 5 and 10 μ g, from left to right) from differentiated P19 cells were incubated with the labelled oligonucleotide probe. Specific protein-DNA complexes are indicated by arrowheads and labelled A-F. (B) Nucleotide sequence around the -93/-27 region of the mouse ERK2 promoter. Potential *cis*-acting sequences are boxed. (C) Identification of the proteins binding to the -93/-51 and -72/-27 probes by antibody supershift. EMSA was performed with 2.5 μ g of differentiated P19 cell nuclear extracts preincubated with 2 μ g each of anti-(NF-YA) (α -NF-YA) (lanes 2 and 7), anti-Sp1 (α -Sp1) (lanes 3 and 8) or anti-Sp3 (α -Sp3) antibodies (lanes 4 and 9) or with various combinations of these antibodies (lanes 5 and 10-13), as indicated above each lane. The positions of protein-DNA complexes are indicated by arrowheads. (D) EMSA using mutant probes. A substitution mutation was introduced into the CCAAT box (lane 3) and GC box (lanes 2 and 5) to abolish binding of the corresponding transcription factors to the -72/-27 (lanes 1-3) and -93/-51 (lanes 4 and 5) probes. Lanes 1 and 4, wild-type (wt) probes.

probe was higher than that with the wild-type probe (Figure 2D, compare lanes 1 and 2). It was therefore suggested that NF-Y and Sp3/Sp1 do not bind to the CCAAT box and GC(I) in a co-operative manner but are mutually exclusive. The CCAAT box mutation also eliminated the minor complex C1. Although we did not examine the identity of complex C1, it could represent a distinct CCAAT box-binding protein other than the prevalent NF-Y, NF-I, C/EBP and CP2, because the competitors representing binding sites for these transcription factors failed to compete for complex C1 (results not shown).

These results together indicate that NF-Y bound to the CCAAT box and that Sp3 predominantly occupied GC(I) and GC(II), whereas Sp1 also bound to these GC boxes but with lesser affinity.

Evaluation of functional importance of the CCAAT box and GC boxes by using mutant reporter constructs

To assess the functional importance of the CCAAT box and of GC(I) and GC(II), we generated a series of mutant promoter

Figure 3 Promoter activity of the CCAAT and GC box mutants

Reporter constructs bearing single or double mutations in the CCAAT box, GC(I) or GC(II) were transiently transfected in undifferentiated P19 cells. The cells were treated with retinoic acid for 3 days and assayed for CAT activity. The CAT activity was normalized for β -galactosidase activity and is represented as a percentage of that of the wild-type pCAT-B(-148/+98) construct. Results are means $\pm$ S.E.M. for at least five transfections.

constructs and assayed for the promoter activity in differentiated P19 cells. The same mutations employed in the EMSA were introduced into the CCAAT box and two GC boxes in pCAT-B(-148/+223) and pCAT-B(-148/+98) in possible combinations. As shown in Figure 3, the disruption of the CCAAT box, GC(I), or GC(II) in pCAT-B(-148/+223), decreased the promoter activity by 57–63%. The promoter activities of double mutants, possessing only one intact element, were similar to each other, 8–12% of the wild-type construct. The CAT activities of the single mutants were greater than the sum of those of the corresponding double mutants; a similar tendency was seen in the CAT activities between the wild-type construct and single mutants, suggesting that a moderate synergy was working between the elements. We also examined effects of these mutations in pCAT-B(-148/+98). The single mutations in pCAT-B(-148/+98) decreased the activity by 31–54% in comparison with wild-type pCAT-B(-148/+98); the double mutants had 12–19% of the activity of the wild-type construct. We observed similar levels of effects conferred by each individual mutation. These three elements therefore seem to have equivalent positive effects on the promoter activity, at least in differentiated P19 cells.

Dominant-negative NF-YA suppresses the promoter activity

We tested effects of the dominant-negative form of NF-YA to establish the role of NF-Y in the ERK2 promoter (Figure 4). The

Figure 4 Effects of dominant-negative NF-YA protein expression on the activity of the wild-type and GC box mutant ERK2 promoter

CHO cells were co-transfected with 300 ng of the wild-type (wt) reporter construct pCAT-B(-148/+223) or one of the corresponding GC box mutant reporter constructs [GC(I) mut, GC(II) mut] in conjunction with the indicated amounts of wild-type (grey bars) or dominant-negative (black bars) NF-YA expression plasmid. Hatched bars represent the CAT activity of each construct without the NF-YA expression plasmids. Results are means $\pm$ S.E.M. for three experiments.

mutant NF-YA, NF-YAm29, which has three amino acid substitutions at the C-terminal region of the subunit, is impaired in its DNA binding and acts as a dominant-negative mutant by sequestering the NF-YB/YC subunits into a defective complex [16]. Along with small (10 ng) or large (50 ng) amounts of either the wild-type NF-YA or NF-YAm29 expression plasmid, CHO cells were co-transfected with 150 ng of the wild-type pCAT-B(-148/+223) reporter plasmid or one of the GC box mutants, pCAT-B(-148/+223) GC(I) mut and pCAT-B(-148/+223) GC(II) mut. Contrary to our expectation, the expression of NF-YAm29 had no effect on the promoter activity conferred by the wild-type reporter. With large amounts, both NF-YA and NF-YAm29 plasmids similarly decreased the promoter activity by 12–25%, not only in co-transfections with the wild type but also in those with the GC box mutant reporters. This partial inhibition of the reporter activity, probably due to competition for transcription factors between the reporter and expression plasmids, has been observed previously in other NF-Y-dependent promoters [23,24]. As the simian virus 40 promoter that drives the expression plasmid was not thought to depend on NF-Y, the transcription factor competed for could be Sp1 or Sp3.

Interestingly, in contrast, NF-YAm29 expression with the GC box mutants significantly lowered promoter activity in comparison with wild-type NF-YA expression. The inhibitory effect was more pronounced in the GC(II) mutant than in the GC(I) mutant. A small amount of NF-YAm29 plasmid was sufficient to give an apparent inhibition of the promoter activity of the GC(II) box mutant, whereas a comparable inhibition of that of the GC(I) mutant required a large amount of NF-YAm29 plasmid. These results confirmed, together with the reporter assay examining the CCAAT box mutant (Figure 3), a role for NF-Y in activating the ERK2 promoter.

DISCUSSION

Here we have reported that a CCAAT box and two bilateral GC boxes are the principal positive *cis* elements in the mouse ERK2 promoter, and identified the corresponding responsible tran-

scription factors. The trimeric transcription factor NF-Y binds to the CCAAT box; Sp3 predominantly binds to the GC boxes.

The CCAAT sequence and its inverted counterpart, ATTGG, are found in approx. 30 % of both TATA-containing and TATA-less eukaryotic promoters at positions between -60 and -100 [25,26]. Among a number of CCAAT-binding proteins isolated so far, the heterotrimeric transcription factor NF-Y (also called CBP and CP1) has been shown to be an essential transcription factor responsible for the regulation of most CCAAT box-containing promoters [26-28]. The GC box is a classic *cis* element that occurs frequently in vertebrate promoters [25]. The Sp family of transcription factors binds to GC boxes and is responsible for transcriptional regulation via these regulatory elements [29].

Consistent with the results reported here for ERK2 is the observation that many other NF-Y-regulated promoters involve Sp1/Sp3, which bind to the GC box(es) located near the CCAAT box [26]. In certain promoter contexts, alignment of the CCAAT box and other *cis* elements are situated to give rise to an appropriate topology of transcriptional machinery [30]. In this regard, it would not be coincidental that some of the CCAAT boxes in the GC box-containing promoters, including the ERK2 promoter, occur at -64 from the transcription start site [18,31]. However, the spatial and functional relationships between NF-Y and Sp1/Sp3 are quite different between the promoters that contain both of the *cis* elements. NF-Y and Sp1 often enhance their DNA binding co-operatively and thereby activate transcription synergistically [32]. In contrast, the binding of Sp1/Sp3 to the proximal GC box could inhibit NF-Y binding as in the *K⁺ channel Kcnj2* promoter [19]. In the ERK2 promoter we did not observe a synergistic binding of Sp3 and NF-Y. When assessed from the levels of binding to the wild-type compared with mutant EMSA probes, they are somewhat inhibitory of each other for binding, presumably owing to mutual steric hindrance. However, as demonstrated by the mutant reporter assay, the two elements showed a moderate synergy in transcriptional activation. A synergistic mechanism working via other than co-operative binding could account for this observation [33].

In the promoter of the gene for ERK1, the most closely related MAP kinase to ERK2, there exist many potential GC boxes but no CCAAT box [5]. It is therefore possible that NF-Y could be important in the differential expression patterns of these two ERK genes. Although the three subunits of NF-Y are expressed, quite ubiquitously and constitutively, as with ERK2, recent studies have shown regulation of the binding activity of NF-Y during cell differentiation and proliferation owing to a change in NF-YA protein levels [34-37]. It would be interesting to examine whether the expression of ERK2 is regulated in accordance with these changes in NF-Y binding activity.

Both GC boxes in the ERK2 promoter preferentially bind Sp3 rather than Sp1 and activate transcription equally. Although the role of Sp1 as a ubiquitous activator is well established, that of Sp3 has been elusive, because Sp3 seems to act as both activator and inhibitor in different studies. Given their comparable affinities for GC box sequences, they seem to exert complex transcriptional regulation through competition for GC boxes. However, it now seems that the activating role is exclusively attributed to full-length Sp3 protein, whereas N-terminally truncated Sp3, whose translation starts internally, is responsible for the GC box-mediated inhibition [17]. This finding is consistent with the stimulatory role of Sp3 binding to both GC boxes in the ERK2 promoter, because the amount of the full-length Sp3 bound to the GC boxes is much greater than that of the truncated protein. Although the relative amounts of Sp1 and the two isoforms of Sp3 protein occupying these GC boxes might reflect their

expression levels, it seems likely that additional factors, such as interaction with nearby transcription machinery and the sequences adjacent to the GC box, affect the binding of Sp proteins to the elements.

With regard to the function of NF-Y, the effect of a dominant-negative mutant of the NF-YA subunit on ERK2 mutant reporter construct is intriguing. Although the NF-YAm29 had no effect on the wild-type promoter, it decreased promoter activity in GC box mutants, the effect being more pronounced on the GC(II) mutant than on the GC(I) mutant. This suggests that the dominant-negative trait of NF-YAm29, impaired binding of DNA, might be complemented by the binding of Sp3 to the adjacent GC boxes. The difference in this effect between GC(I) and GC(II) mutants could arise either from the alignment of NF-Y and Sp3 or from the asymmetric nature of NF-Y. The functional interaction of NF-Y and Sp3 could include a direct protein interaction similar to that observed between NF-YA and Sp1 [38]. Further detailed analyses will be necessary to elucidate the functional interaction of NF-Y and Sp proteins in transcription.

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