The Mouse Extracellular Signal-regulated Kinase 2 Gene

GENE STRUCTURE AND CHARACTERIZATION OF THE PROMOTER*†

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ERK2 (extracellular-signal-regulated kinase 2, also known as p42 mitogen-activated protein kinase) is an integral member of the mitogen-activated protein kinase cascade that is crucial for many cellular events such as proliferation and differentiation. Here, we determined the genomic organization of the Erk2 gene and characterized its promoter. The Erk2 gene spans over 60 kilobases, and the coding region is split into eight exons. In the coding region, exon-intron organization was exactly conserved between the two mouse genes for ERK2 and ERK1 except one junction shifted by one nucleotide. Primer extension and S1 nuclease analyses identified two major transcription start sites located at −219 and −223 relative to the translation start site. The 5′-flanking sequence lacked TATA box but contained a CCAAT box located approximately 60 base pairs upstream of transcription start sites. Sequencing of the 5′-flanking region also revealed potential cis-acting elements for multiple transcriptional regulatory factors including Sp1, z/268, Ets, CREB, and PuF sites. The promoter activity of the 5′-flanking region was examined using chloramphenicol acetyltransferase as a reporter gene. Transient transfection experiments using Chinese hamster ovary cells defined a maximal promoter activity in a 371-base pair region immediately upstream of the translation start site. Furthermore, we demonstrated, using mouse P19 embryonal carcinoma cells, that this 371-base pair sequence is likely to be sufficient to confer the transcriptional activation of the ERK2 promoter during the retinoic acid-induced differentiation of P19 cells.

The mitogen-activated protein (MAP)1 kinase cascade is activated by a myriad of proliferation- and differentiation-inducing stimuli (1–6). The MAP kinase pathway is utilized by broad organisms, including slime mold, yeast, fly, plants, and mammals, in a well conserved fashion. Following the activation of receptor tyrosine kinases and G-protein-coupled receptors, the signal is transmitted sequentially through an array of proteins including Ras, MAP kinase kinase kinases (Raf, MEK kinase, Mos), and MAP kinase kinase (MEK). MAP kinases are then phosphorylated on the tyrosine and threonine residues by dual-specificity kinase MEK. The activated MAP kinases translocate to the nucleus where the signal is converted to change transcriptional activity. MAP kinase activation also leads to phosphorylation of various proteins including membrane receptors, other protein kinases, cytoskeletal proteins, and regulatory enzymes.

ERK1 (extracellular signal-regulated kinase 1) and ERK2, known as 44- and 42 kDa-protein, respectively, were the first vertebrate MAP kinases to be described (7–9) and are the most closely related isoforms among the increasing MAP kinase multigene family (10) sharing approximately 90% identity in the amino acid sequence (9). These two kinases are similar in many aspects, such as ubiquitous tissue distribution (9, 11, 12), sensitivity to activation by MEK (13), and substrate specificity (2). Although ERK1 and ERK2 could be functionally redundant, it seems more likely that distinct patterns of gene expression impart specific biological role(s) to each ERK isoform in accordance with selective activation of the ERK isoforms; various stimuli selectively activate ERK2 in different cell contexts (14–20). Distinct regional distribution of ERK1 and ERK2 in brain structures (9, 21, 22) appears to result from unique transcriptional regulation on each gene.

Studies examining the specificity of growth factor signaling have suggested that the duration of the MAPK cascade activation makes cells decide about proliferation versus differentiation (4). Thus, proper regulation of Erk gene expression may be required for determining cell response by restricting the potential for activation of the corresponding cascade. However, little is known about the transcriptional regulation of ERKs. Early works have demonstrated an increased expression of ERK2 mRNA in the developing brain with concomitant change in the protein level (9). It was also shown that neuronal differentiation of P19 mouse embryonal carcinoma cells is associated with an increase in ERK2 mRNA (9). Analysis in cultured kidney mesangial cells has suggested that the regulation of the Erk2 gene expression resembles that of an immediate early gene (23).

To further elucidate the mechanisms that underlie these regulatable aspects of the Erk2 gene, we cloned the Erk2 gene and identified the promoter region. Determining the organization and promoter sequence of the Erk2 gene will enable a comparative analysis with those of the previously isolated mouse Erk1 gene (24). It would also help to investigate the possible occurrence of alternatively spliced transcripts that could give rise to different forms of ERK2 protein observed in human tissues (12). A comparison of structural features between the genes for ERK1 and ERK2 might provide insights into evolution of these closely related kinase genes after gene duplication. In the present study, we determined the structure and 5′-flanking sequence of the mouse Erk2 gene and characterized its promoter.

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1 The abbreviations used are: MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; ERK(s), extracellular signal-regulated kinase(s); MEK, ERK kinase or MAP kinase kinase; bp, base pair(s); kb, kilobase pair(s); PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; CHO, Chinese hamster ovary; EC embryonal carcinoma; RA, retinoic acid; CREB, cAMP response element-binding protein.
**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction and modifying enzymes were purchased from Life Technologies, Inc., New England Biolabs, Inc., Promega Corp., and Boehringer Mannheim. Radiolabeled nucleotides and [3H]chloramphenicol were from NEN Life Science Products. Nondenatation kit was from Pharmacia Biotech Inc. Cell culturing media were from Life Technologies, Inc. Bovine serum and fetal bovine serum were from JRH Biosciences.

**Northern Blot Hybridization Analysis**—For Northern blot analysis, 5 μg of poly(A)+-rich RNA from mouse brain and lung were fractionated on a formaldehyde, 1% agarose denaturing gel and transferred to Hybond-N+ nylon membrane (Amersham Corp.). The blot was prehybridized in 50% formamide, 6× SSPE, 1% SDS, 2× Denhardt’s solution and 100 μg/ml denatured herring sperm DNA at 42°C for 2 h. Hybridization was performed at 42°C for 10 h with 2× 106 cpm of labeled probe/ml in the same solution as prehybridization except for omitting Denhardt’s solution. Washing was carried out 3 times in 1× SSC and 0.1% SDS at room temperature for 10 min and then in 0.1× SSC and 0.1% SDS at 65°C for 45 min. The probe used was mouse Erk2 cDNA fragment (consisting of 60 bp of the 5′-untranslated region and 5′ 485 bp of the coding region; Ref. 25) labeled with [α-32P]dCTP by the random primer method (26); the Sph I terminus coding region showed the least sequence homology between ERK1 and ERK2. Preliminary dot blot hybridization analysis confirmed that under this condition, the Erk2 cDNA probe hybridized with more than 1,000-fold selectivity toward Erk2 cDNA sequence over ERK1 cDNA sequence. Autoradiography was done at −80°C with an intensifying screen for 3 days.

**Structural Analysis of the Erk2 Gene**—A P1 clone isolated from a cloned human embryonic stem cell library was obtained from Genometech Systems (St. Louis, MO). The PCR primers used for screening were PS-1U (5′-AAACAGGTGGTGGTTATCA-3′) and PS-1D (5′-ATT-GAGTCCAAATGATGTTCTC-3′) which amplify a 122-bp product between nucleotides 152 and 273 (translation start site as +1) of the mouse Erk2 cDNA sequence (25). To obtain a higher yield of P1 plasmid DNA, the P1 plasmid in Escherichia coli N53529 was transferred to E. coli strain S1361 by production of a P1 transducing phage (27). P1 plasmid was purified by the plasmid preparation method 1 suggested by Sambrook et al. (9).

The clone 7278 P1 plasmid was subjected to restriction site mapping. A 4.5-kb EcoRI fragment containing the Erk2 gene 5′-flanking region was identified by probing the EcoRI digests with 32P-labeled oligonucleotide probe U3 (5′-CCCGAAGGCTGAGCCGAACGC-3′) which represents the 5′ extremity of the known mouse Erk2 cDNA sequence. Plasmid pBS-E4.5 was made by subcloning this fragment into pBlue-script (Stratagene). To analyze the gene structure, blots of restriction plasmid DNA, the P1 plasmid in Escherichia coli N53529 (25) were sequenced to verify the orientation and cloning junction of the plasmid. All constructs were transfected by the lipofection method using porter constructs were transfected in pCAT-Basic (Promega). As a positive control, prSV CAT (30) was used. Plasmid pCAT-B(-2425/+100) was constructed by inserting a 2523-bp SpII/H新华 fragment of pBS-E4.5 between the SpII/H新华 sites (with PstI site blunted) of the pCAT-Basic polylinker. To construct pCAT-B(-2425/-100), 2.6 kb of the 5′-flanking region immediately upstream of the translation start site, we generated a 570-bp PCR fragment encompassing nucleotides −338 to +123 with an additional SaI site at its 3′ end. Primers used were as follows: 5′-primer, 5′-CTCCAGGGCTCCGTTGCTGGT- GTG-3′; and 3′-primer, 5′-AGATGTCAGGCTGTGCCACGACGCC-3′. Plasmid pBS-f0.57 was made by subcloning this fragment into pBluescript, and the nucleotide sequence of the insert was confirmed by dyeoxy sequencing. Then pCAT-B(-2425/-100) was digested with EcoRI and SaI and inserted with a 398-bp fragment, which was derived from the digestion of pBS-f0.57 at the corresponding HindIII site and the SaI site in the pCAT-B(-2425) (number in parentheses with a construct represents the position of the 5′ end of the insert relative to the upstream major transcription start site). A series of 5′-deleted reporter plasmids was constructed using convenient restriction sites. Plasmid pCAT-B(-2425) was cut with HindIII and filled with Klenow and then cut with AwaI, BstEII, SacI, DrosIII, and NcoI to make pCAT-B(-1156), pCAT-B(-781), pCAT-B(-542), pCAT-B(-366), and pCAT-B (+99), respectively. The linearized plasmid blunt cut the protruding end and was self-ligated. Plasmid pCAT-B(-1235) was created by self-ligation of HindIII-cut pCAT-B(-2425). Other 5′-deleted plasmids contain EcoO109f/SalI (pCAT-B(-219)), Eco47III/SaI (pCAT-B(-174)), and Eco47III/HindIII/SalI (pCAT-B(-42)), and BglII/SalI (pCAT-B(+29)) fragments derived from pBS-f0.57 (with their 5′ ends blunted) within the HindIII (blunted) and SaI sites of the pCAT-basic polylinker. All constructs were sequenced to verify the orientation and cloning junction of the insert.

*p72 Transfection and CAT Assay*—CHO-K1 cell was maintained in Ham’s F-12 medium supplemented with 10% fetal bovine serum. Reporter constructs were transfected by the lipofection method using pFx-4 lipid (Invitrogen) according to the protocol recommended by the supplier. Briefly, 3 × 105 cells were seeded on 60-mm dishes 1 day before transfection. Using 15 μg of pFx-4, 4 μg of reporter construct was cotransfected with 1 μg of pCH110 (Pharmacia). The internal standard plasmid, pCH110, which bears the lacZ gene under the control of SV40 early promoter, was included to normalize transfection efficiencies. Transfection was carried out for 4 h, and the medium was changed to the complete medium. After a 48-h incubation, the cells were harvested, and cell extracts were prepared in 1 × reporter lysis buffer (Promega). The cell extracts were assayed for CAT activity using a phase extraction method (31), and β-galactosidase activity of CHO cell extracts was determined using the chromogenic substrate o-nitrophenyl β-D-galactopyranoside (32).

P19 mouse embryonic carcinoma (EC) cells were cultured in α-minimal essential medium supplemented with 7.5% bovine serum and 2.5% fetal bovine serum (32). Activities of transfected reporter genes in undifferentiated and differentiated P19 cells were evaluated as follows. One day before transfection, 7 × 105 undifferentiated P19 cells were seeded on 60-mm dishes. Transfection was carried out in serum-free medium.
Expression of Mouse ERK2 mRNA—Previous experiments analyzing ERK2 mRNA transcripts have been performed using RNA isolated from rat (9) and human (12) tissues. To characterize the mouse gene for ERK2, we examined an expression pattern of ERK2 mRNA by Northern hybridization analysis using poly(A)⁺ RNA from mouse tissues. Hybridization and washing of the blot were carried out under stringent conditions to avoid cross-hybridization of the ERK2 cDNA probe with the ERK1 transcripts. As has been reported by Boulton et al. (9), who used rat total RNAs, mouse ERK2 mRNA was expressed at much higher levels in brain than in the peripheral lung tissue (Fig. 1). In rat tissues, three distinct ERK2 transcripts were identified at different ratios among tissues. Similarly, multiple ERK2 mRNA transcripts were expressed in the human (12). As shown in Fig. 1, mouse ERK2 transcripts displayed several isoforms as do rat and human ERK2 transcripts. Four ERK2 transcripts, 8.1, 5.3, 3.1, and 2.3 kb in size, were found in brain. The lung transcripts showed a distinct pattern. The 8.1- and 5.3-kb transcripts were expressed at barely detectable amounts. In contrast, discrete transcripts of 3.6 and 3.0 kb were observed in the lung at higher levels. The 1.9-kb transcript was detected only in lung. These findings confirm that the characteristics of ERK2 mRNA transcripts, namely the abundance in brain and the tissue heterogeneity, are also observed in mouse tissues.

Structure of the Erk2 Gene—We analyzed one P1 clone, which was isolated from a mouse embryonic stem cell P1 library by screening with PCR, and found it contained an 80-kb insert and covered all of the exons encoding the ERK2 protein sequence. Through a combination of restriction mapping, Southern blot, and sequencing analyses, the exon-intron organization of the Erk2 gene was elucidated as shown in Fig. 2 and Table I. The entire protein-coding sequence was identical to the reported cDNA sequence (25). The gene spanned over 60 kb, and the protein-coding region was divided into eight exons. Exon 1 consisted of approximately 220 bp of the 5′-untranslated region and 113 bp of the coding sequence. Exon 8 contained the 3′-coding and untranslated region. The nucleotide sequences of the exon-intron boundaries (Table I) conformed to the consensus sequence for splice donor and acceptor sites (GT-AG rule; Ref. 33). Internal exons ranged in size from 110 to 190 bp. Introns ranged from 0.2 kb (intron E) to the large 32 kb (intron A). In the coding sequence, positions of the splice junctions were in good agreement with those of the previously cloned Erk1 gene (24), except the intron D splicing site. The 5′ splice site of intron D was shifted by one nucleotide toward intron A. A 4.5-kb EcoRI genomic fragment containing the 5′-flanking region of the Erk2 gene was identified by Southern analysis and subcloned into pBluescript as described under “Experimental Procedures.” The plasmid, pBS-E4.5, was used for further analysis of the 5′-flanking region.

Determination of Transcription Start Sites—To determine the transcription start site(s) of the Erk2 gene, primer extension analysis and S1 nuclelease protection assay were performed in combination. For primer extension analysis, two radiolabeled oligonucleotides were used in the extension reaction. First extension analysis used oligonucleotide PE-1, which is proximal to the translation start site (complementary to bases −25 to −53), the adenine residue of the ATG start codon is referred to as +1), as a primer. When extension was carried out with poly(A)⁺ RNA from mouse brain, three major products were observed at bases −219, −223, and −330, with minor products at −205, −208, −226, and −278 (Fig. 3A). In the reaction using lung poly(A)⁺ RNA, the product at −330 was not observed. In the second extension reaction employing the distal primer PE-2 (complementary to bases −129 to −158), the extended products terminated at two major sites, −219 and −223, and three minor sites, −205, −208, and −226 (Fig. 3B). The reactions with brain and lung poly(A)⁺ RNA gave the same products, and the sites coincided with those of the reaction using PE-1. The two longer products obtained with PE-1 as primer (at −278 and −330) were not observed in the extension reaction with PE-2. They might reflect transcripts with different 5′ ends that were generated from alternative splicing occurring within the 100-bp stretch between PE-1 and PE-2. We cannot exclude the possibility that another 5′-untranslated exon lies in the 5′-upstream region.

S1 nuclease assay was also performed to confirm the results obtained with the primer extension analyses. A 284-nucleotide single-stranded DNA probe was hybridized to brain and lung poly(A)⁺ RNA and digested with S1 nuclease. The protected fragments analyzed on a sequencing gel gave the consistent

**FIG. 1. Northern blot analysis of mouse ERK2 mRNA transcripts.** Poly(A)⁺ RNA was isolated from mouse brain and lung. Each lane contains 5 μg of poly(A)⁺-rich RNA. The blot was hybridized with the radiolabeled ERK2-specific probe under stringent conditions to eliminate cross-hybridization with ERK1 transcripts. The positions of RNA markers are shown at the left.

**FIG. 2. Structure of the Erk2 gene.** Exons are indicated by hatched boxes numbered 1–9. Sites for the restriction endonuclease Kpn1 (K) and XhoI (X) are indicated.
pattern observed in the primer extension using PE-2, with two major protected fragments at −220 and −223 and three minor fragments at −205, −209, and −226 (Fig. 3B). The minor signals at −205 and −209 were weak and only visible after longer exposure (data not shown). The two nuclease-resistant fragments (at −209 and −220) that are longer than the corresponding primer-extended products seemed to have 3′ signals at relatively resistant to S1 nuclease digestion.

Taken together, these findings revealed that the Erk2 gene is transcribed from multiple start sites with the major sites being mapped at bases −219 and −223. The context of the major start site at −223 agrees well with the eukaryotic initiator consensus sequence (YAY, NWY; Ref. 34), although the preceding C residue is utilized as the initiation site instead of the authentic A residue (Fig. 3C).

Potential Regulatory Elements in the 5′-Flanking Sequence of the Erk2 Gene—To identify sequence elements that may be involved in transcriptional regulation, we determined the nucleotide sequence of the 5′-flanking region contained in pBS-E4.5 (Fig. 4). The 400-bp stretch, with the major transcription start sites set on center, had high GC content (68%). No canonical TATA box was found around the transcription start sites. A CCAAT box was evident at 60 nucleotides upstream from the major transcription start site. Six Sp1 (35) binding core sequences were distributed around the transcription start sites; two of the Sp1 sites were located upstream of the start site (at −86 and −39 nucleotides relative to the upstream major start site), and four were at positions +11, +98, +104, and +116. Two of the downstream Sp1 sites comprised a part of the zj/268 (also known as Krox-24, Egr-1, NGFI-A; Ref. 36) site (GGGGGGGCG; Fig. 4).

A computer-assisted search revealed multiple sequence elements having homology to known binding sequences for transcription factors. These included two AP-2 sites (consensus sequence: 5′-CCSCRGCCG, Ref. 37) at −180 and +259, two Ets sites (MGGAAG, Ref. 38) at −107 and +24, CREB site (TGACGCTCA, Ref. 35) at −153, PuF site (GGGTGG, Ref. 39) at −236, Antennapedia site (ANNNNCATTA, Ref. 40) at +461, bicoid site (TCTATCCC, Ref. 41) at −503, MyoD site (CANTTO, Ref. 42) at −779, and ELP site (TCAAGGC, Ref. 43) at −786. A comparison by dot matrix analysis of the 5′-flanking sequences between the Erk1 and Erk2 genes revealed no apparent overall homology throughout the 5′-flanking region (data not shown).

Promoter Activity of the 5′-Flanking Region of the Erk2 Gene—To assess promoter activity of the cloned 5′-flanking region by its ability to drive CAT gene expression, we first made a construct by inserting a 2.6-kb fragment, which extends immediately upstream of the translation start site, in front of the CAT gene (pCAT-B−(−2425)). Transient transfection assay was performed using CHO cells, which express high levels of ERK2 protein (11), and the 2.6-kb fragment showed a significant promoter activity (Fig. 5). The CAT activity of pCAT-B−(−2425) was 27% of that driven by the Rous sarcoma virus long terminal repeat (pRSV-CAT), indicating that the Erk2 gene promoter could direct high level transcription. We subsequently examined the effect of a series of 5′ deletions of the 2.6-kb fragment on the promoter activity. As shown in Fig. 5, deletion to base −781 had little effect on CAT activity, whereas progressive deletions to bases −542 to −148 resulted in a gradual increase. The increases indicated the existence of multiple repressive elements in this region. The maximal activity was marked by a pCAT-B−(−148) construct with a 2.5-fold increase over that of pCAT-B−(−2425). Truncation of a 106-bp sequence from pCAT-B−(−148) resulted in more than 85% loss of CAT activity. These results suggested that the 371-bp region between −148 and +223 could direct maximal transcription and that the putative cis-acting elements between −148 and +223, such as Sp1 sites and CCAAT box, are likely essential in basal transcriptional activity of the Erk2 gene promoter.

Activation of the Erk2 Gene Promoter during Differentiation of P19 EC Cells—The earlier study showed an increase in ERK2 mRNA levels during differentiation of mouse P19 EC cells (9). We, therefore, examined whether the isolated promoter sequence is responsible for the transcriptional activation during differentiation of P19 EC cells. Undifferentiated P19 cells were transiently transfected with either pCAT-B−(−2425) or pCAT-B−(−148). The transfected cells were divided into aliquots and left undifferentiated or induced to differentiate in the presence of 1 μM RA (see “Experimental Procedures”). This concentration of RA has been shown to be enough for inducing the neuronal differentiation of P19 cells, and 2-day exposure to RA induces P19 cells into an irreversibly differentiated state (44). The CAT activity was normalized for transfection efficiency with reference to the β-galactosidase activity derived from the cotransfected internal control plasmid. Concerning the normalization, it would be pertinent to note that, as judged from the β-galactosidase activity, expression of the reporter plasmids in the differentiated cells on day 3 was lowered to 16–66% that on day 2. In contrast, both β-galactosidase activity and CAT activities of the undifferentiated cells remained almost constant during days 2 and 3 (data not shown).

As seen in Fig. 6, differentiation of P19 cells was accompanied by a significant increase in transcriptional activity of the ERK2 promoter. After 3 days of RA treatment, the CAT activity of differentiated P19 cells transfected with pCAT-B−(−148) was elevated 3.5-fold over that of the corresponding undifferentiated cells. Similar to the results obtained in CHO cells, pCAT-B−(−148) showed higher activity than pCAT-B−(−2425) in both undifferentiated and differentiated cells. These findings indicated that the 371-bp region between bases −148 and +223 is sufficient to confer the transcriptional activation of the Erk2 gene during the RA-induced differentiation of P19 cells.

### Table I

**Exon-intron boundary sequences of the mouse Erk2 gene**

Exon sequences are shown in uppercase letters and intron sequences in lowercase letters.

| No. | Exon size | Boundary sequences | Intron size |
|-----|-----------|--------------------|------------|
|     | bp        | 5′ boundary | 3′ boundary |           |
| 1.  | 336/332   | GGTGTC gat gagt...tttt ag CTCTGC | A | 32 |
| 2.  | 183       | AGATGT gat atcc...acac ag ATATAT | B | 2.2 |
| 3.  | 190       | CTCAGA gtt cagc...cccc ag ACTGTG | C | 4.9 |
| 4.  | 117       | TCCAGA gtt aaga...tttc ag GTTGTG | D | 1.5 |
| 5.  | 115       | TCCGTA gtt aagc...ttgt ag GTATTC | E | 0.218 |
| 6.  | 182       | CTCAGA gtt aaga...tttc ag GCTGGG | F | 10 |
| 7.  | 110       | GATGAC gtt aaga...tttaa ag GCCATT | G | 2.9 |
| 8.  | 126       | GGTCA gtt atct...tttc ag GACAGAG | H | 5.2 |
The Erk2 (p42 MAPK) Gene

In this study, we determined the genomic structure of the mouse Erk2 gene and identified its promoter. The Erk2 gene is distributed over 60 kb and has 8 exons. The size of the gene is much larger than that of the previously cloned mouse Erk1 gene (24), about 8 kb in length, because of the expansion of the corresponding introns. The exon-intron organization of the coding region is highly conserved between two genes for ERK2 and ERK1. The only difference was seen in intron D with its 5′ splice site shifted by only one nucleotide. All of the protein kinase conserved subdomains (45) are distributed throughout the exons in the same manner as the Erk1 gene; in contrast to the other kinase genes (46, 47) in which some of the kinase subdomains are interrupted by the introns, all of the kinase subdomains of the Erk2 gene are retained in a single exon. Primer extension and S1 nuclease analyses identified two major transcription start sites 219 and 223 bp upstream of the translation start site. Sequence analysis of the 5′-flanking region revealed multiple potential cis-acting elements including Sp1, zif268, Ets, CREB, and PuF sites. TATA box was not found, whereas a CCAAT box was found located about 60 bp upstream of the two major transcription sites. A comparison between the 5′-flanking sequences of the Erk2 and Erk1 genes showed no apparent homology in contrast to the highly conserved gene structure. The divergence in the regulatory sequence is likely to enable a unique expression pattern of each Erk gene by employing distinct combinational assortments of transcription factors.

Since ERKs occupy a pivotal position in MAPK cascades, any change in expression levels of ERK isofrom could be reflected in the amplitude and duration of signaling of the corresponding MAPK module. Accordingly, expression of Erk genes should be strictly regulated to maintain cell function. Thus, to elucidate the transcriptional machinery that modulates ERK2 expression, we analyzed promoter activity of the 5′-flanking region of the gene. The 2.6-kb fragment of the 5′-flanking region showed marked promoter activity when transiently transfected in CHO cells with cat as a reporter gene. Since the sequence analysis of this 2.6-kb region had revealed multiple potential cis-acting elements that are implicated in regulation of developmental- and tissue-specific gene expression, we constructed a series of deletion constructs and examined their promoter activity. Deletion to base −781 had little effect on CAT activity, whereas further progressive deletions to −148 resulted in a gradual increase. This suggests that some of the putative cis-acting elements between bases −781 and −148, namely MyoD, bicoid, Antennapedia, PuF, AP-2, CREB and Ets sites, could mediate a repressive effect on the Erk2 gene expression in CHO cells. The highest CAT activity of pCAT-B(−148) was dramatically reduced by removing of a 5′ 106-bp sequence, suggesting the importance of two Sp1 sites and CCAAT box included between −148 and −42.

Fig. 3. Mapping of transcription start sites of the Erk2 gene. Primer extension and S1 nuclease analyses were performed as described under “Experimental Procedures.” A, primer extension analysis using oligonucleotide PE-1 (located in Fig. 4) as a distal primer. The end-labeled primer was extended on 5 μg of poly(A)+ RNA from mouse brain (lanes 1 and 2) and lung (lane 3) with Superscript II reverse transcriptase. Control reaction included yeast tRNA instead of poly(A)+ RNA (lane 4). Each reaction contained 1 × 10⁶ cpm (lanes 1, 3, and 4) or 2 × 10⁵ cpm (lane 2) of ³²P-labeled primer. Corresponding genomic fragment was sequenced with the same end-labeled primer and electrophoresed alongside to locate the 3′ terminus of extension products (not shown). Numbers at the left indicate the corresponding position of the 3′ terminus relative to the translation start site. B, primer extension and S1 nuclease analyses using oligonucleotide PE-2. Primer extension was carried out using a proximal primer PE-2 (located in Fig. 4) in place of PE-1; lane 4, brain poly(A)+ RNA; lane 5, lung poly(A)+ RNA; lane 6, yeast tRNA. To compare the result of S1 assay directly to that of the primer extension analysis, the same oligonucleotide PE-2 was radiolabeled at the 5′ terminus and used to synthesize a single-stranded DNA probe for the S1 assay. The probe was hybridized to 5 μg of poly(A)+ RNA from brain (lanes 2 and 3) and lung (lane 1) and treated with 300 units (lanes 1 and 3) or 1,000 units (lane 2) of S1 nuclease at 37 °C for 30 min. A sequencing ladder of the corresponding genomic fragment, which was primed with the same ³²P-labeled oligonucleotide, was electrophoresed alongside. The positions at which the products terminated are assigned as in A. The primer extension experiments using either PE-1 (A) and PE-2 (B) gave concordant results. C, transcription start sites of the Erk2 gene identified by primer extension and S1 analyses. Arrowheads indicate the start sites identified by primer extension analyses. The sites identified by S1 assay are marked with circles. The major and minor start sites are represented by closed and open symbols, respectively.
The marked increase in the ERK2 promoter activity during the RA-induced differentiation of P19 EC cells agreed well with the results obtained by the Northern hybridization experiment (9). The proximal 371-bp region was sufficient to confer the responsiveness, and the promoter activity had been elevated significantly by day 2. Since the neural differentiation of P19 cells was shown to be induced irreversibly by 2-day treatment with RA (44), it is highly likely that the increased expression of ERK2 is critical for the process. Supporting this idea is a current model that the neuronal differentiation of PC12 pheochromocytoma cells is determined by the sustained activation of the ERK pathway (4). In the nervous system, expression of ERKs shows developmentally regulated and restricted patterns (9, 21, 22). A role of ERK2 for modulation of neural activity has been suggested in hippocampal long term potentiation (20) which is associated with the induction of a set of immediate early genes. Taking these observations together, it seems that the transcriptional control of the Erk2 gene is essential for development and function of the nervous system.

The previously isolated kinase genes mostly lack both the TATA and CCAAT boxes or have them at a nonstandard position (47–50). Together with other common features such as multiple transcription start sites, GC-enriched 5'-flanking sequence, and multiple Sp1 sites, these kinase genes are considered as housekeeping types. The Erk2 gene promoter is unusual in that it has characteristics of both housekeeping genes and regulated genes. While this promoter has the latter three features common to housekeeping genes, it also contains a canonical CCAAT box and potential cis-acting elements that act in various developmental- and tissue-specific contexts. Indeed, the Erk2 gene promoter is inducible as demonstrated in our experiment using P19 cells. A similar promoter has been described in the aspartate aminotransferase gene (51) which is expressed not only constitutively but also in a hormonally regulated manner. In the aspartate aminotransferase gene, two CCAAT boxes, which accounted for the basal activity of the promoter, interact with a different set of CCAAT box-binding proteins depending on the tissue examined. It seems that constitutive activity of the promoter may correlate with the ubiquitous occurrence of the different CCAAT box-binding proteins.

**Fig. 4.** Nucleotide sequence of the Erk2 gene, the 5'-flanking region, exon 1, and a part of intron A. The upstream major transcription start site is numbered as +1. Exon sequences are represented by uppercase letters, and intron sequences are represented by lowercase letters. The major transcription start sites are represented by bent arrows. Open arrowheads mark the minor start sites. Oligonucleotides PE-1 and PE-2, which were used in primer extension and S1 nuclease analyses, are located by horizontal arrows. Potential cis-acting elements are boxed.

**Fig. 5.** Functional analysis of the mouse Erk2 gene promoter. A series of the 5'-deleted ERK2 promoter constructs were transfected in CHO-K1 cells. CAT activity is represented in arbitrary units normalized for β-galactosidase activity. The results shown are the average of at least four independent transfections with the S.D. indicated by error bars.
including the CCAAT/enhancer-binding protein (C/EBP) family (52), CTF/NF-I (35), and NF-Y/CP1/CBF-related proteins (53).

On the other hand, differences in combinations of these transcription factors may be reflected in tissue-specific or regulated activity of the promoter. Likewise, the unique property of the Erk2 gene promoter might make it possible to compromise different regulatory requirements for widespread expression and responsiveness for various stimuli; the transcriptional regulation of the Erk2 gene might involve multiple CCAAT box-binding proteins as in the aspartate aminotransferase gene. Further experiments are needed to precisely define the cis-acting elements and transcription factors involved in regulating the Erk2 gene expression.

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