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ERF: Genomic organization, chromosomal localization and promoter analysis of the human and mouse genes

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***ERF* (*E*ts2 *R*epressor *F*actor) is a ubiquitously expressed ets-domain protein that exhibits strong transcriptional repressor activity, has been shown to suppress *ets*-induced transformation and has been suggested to be regulated by MAPK phosphorylation. We report here the sequence of the mouse gene, the genomic organization of the human and the mouse genes, their chromosomal position and the analysis of the promoter region. Genomic clones encompassing either the human *ERF* or the mouse *Erf* gene were isolated and utilized to define their molecular organization. The gene in both species consists of 4 exons over a 10 kb region. Utilizing FISH, somatic cell hybrids and linkage analysis, we identified the chromosomal position of *ERF* on human chromosome 19q13.1 and on its syntenic region in the mouse, on chromosome 7. Sequence analysis of the mouse gene indicated a 90% identity to the human gene within the coding and promoter regions. The predicted Erf protein is 98% identical to the human protein and all of the identifiable motifs are conserved between the two proteins. However, the mouse protein is three amino acids longer (551 *versus* 548 aa). The area surrounding the region that is homologous to the 5′ end of the human cDNA can serve as a promoter in transfection into eukaryotic cells. This region is highly conserved between the mouse and the human genes. A number of conserved transcription factor binding sites can be identified in the region including an ets binding site (EBS). Interestingly, removal of a small segment that includes the EBS, seriously hampers promoter function, suggesting the ERF transcription may be regulated by *ets*-domain proteins.**

Keywords: ets; transcriptional repressor; genomic organization; promoter

Introduction

The *ets* family of genes was originally discovered by its homology to the avian transforming virus E26 (Nunn *et al*., 1984; Bister *et al*., 1982) and is characterized by a conserved DNA binding domain (Watson *et al*., 1988; Karim *et al*., 1990) which recognize the GGA A/T motif (Macleod *et al*., 1992; Wasylyk *et al*., 1993; Werner *et al*., 1995; Kodandapani *et al*., 1996). *Ets* genes have been found throughout the metazoan evolution (Lautenberger *et al*., 1992; Laudet *et al*., 1993; Degnan *et al*., 1993) which is suggestive of their fundamental role. They have been implicated in cellular proliferation and tumorigenesis (Macleod *et al*., 1992; Wasylyk *et al*., 1993; Seth *et al*., 1992; Janknecht and Nordheim, 1993) and have been suggested to be involved in the regulation of key cellular genes as c-*fos* (Karin, 1994; Treisman, 1994), c-*myc* (Roussel *et al*., 1994), Rb (Savoysky *et al*., 1994) and *ETS2* (Mavrothalassitis and Papas, 1991). They have been found to cooperate with other cellular transcription factors in early mitogenic response (Treier *et al*., 1995; Sieweke *et al*., 1996) and have been suggested to be downstream effectors in the *ras*/MAPK signalling pathway (Janknecht and Nordheim, 1993; Yang *et al*., 1996; Janknecht, 1996; Gille *et al*., 1992; Hill *et al*., 1993; Marais *et al*., 1993; O'Neill *et al*., 1994; Rao and Reddy, 1994; Brunner *et al*., 1994) and regulated by MAPK. The data suggesting a possible association of *ets* genes with proliferative processes have been supported by the identification of *ets* genes at chromosomal breakpoints, which result in *ets* gene rearrangement in certain human malignancies. *Fli1* has been identified in 11;22 translocation of Ewing's Sarcoma (May *et al*., 1993; Delattre *et al*., 1992) and peripheral neuroepithelioma (Hromas *et al*., 1993; Hromas and Klemsz, 1994). The *ERG* gene in 21;22 translocation in Ewing's Sarcoma (Sorensen *et al*., 1994) and 16;21 translocation in AML (Shimizu *et al*., 1993; Panagopoulos *et al*., 1994), the ETV1 in 7;22 Ewing's Sarcoma translocation (Jeon *et al*., 1995) and the TEL gene in 5;12 and 22;12 translocations in myoproliferative disorders (Buijs *et al*., 1995; Wlodarska *et al*., 1995). Finally, it recently has been shown that overexpression of *ets2* in mice can partially mimic the Down Syndrome phenotype (Sumarsono *et al*., 1996).

ERF is a novel member of the *ets* family that has been isolated by its interaction with the *ETS2* promoter (Sgouras *et al*., 1995). It has no similarity to other *ets* genes outside the DNA-binding domain; the family member most similar to *ERF* is PE-1 (Klemsz *et al*., 1994). *ERF* is ubiquitously expressed, exhibits strong transcriptional repressor activity and has been shown to be regulated by phosphorylation throughout the cell cycle and during mitogenic stimulation. Furthermore, is capable of suppressing *ets* and *fos* induced tumorigenicity in the NIH3T3 system and may act as a tumor suppressor gene.

To further analyse *ERF's* function and its possible implication in malignancies, we isolated both the human and mouse genes and analysed their structure and organization. Both genes have identical intron/exon

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boundaries although the intron sizes are slightly different. The genes are 90% indentical within their coding region at the nucleotide level and 98% identical at the amino acid level. Interestingly, most of the differences appear at the carboxy terminus, which harbors the transcriptional repressor domain. Both genes are driven of a promoter with a high GC content; this is consistent with their ubiquitous expression. Interestingly, the proximal promoter region between the two species is as homologous as the rest of the coding region, i.e. about 91%. The human gene is localized on chromosome 19q13.1, while the mouse gene in the syntenic region on mouse chromosome 7. The chromosomal position of *ERF* suggests a possible implication in certain human malignancies and makes it a target for future investigation.

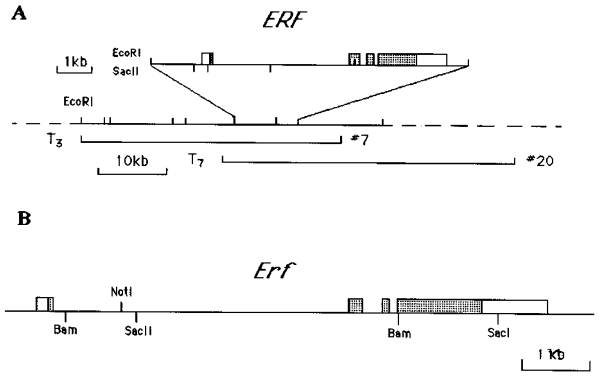

Figure 1 Genomic organization of the human (**A**) and mouse (**B**) *erf* gene. The exons are indicated by boxes and the shaded areas represent the coding regions

Results

Isolation and characterization of ERF *gene from human and mouse*

We utilized the *ERF* cDNA to screen both a human cosmid library (Strategene, La Jolla, CA) and a mouse C127 library (Stratagene, La Jolla, CA). Two overlapping cosmid clones were isolated for the human gene and a phage clone for the mouse gene (Figure 1) that contained the entire *erf* gene. The intron/exon organization of both genes was determined by restriction mapping and dideoxy sequencing utilizing primers derived from the human cDNA sequence as well as new ones derived from the determined mouse sequence. The gene in both species consists of 4 exons, the 2nd and 3rd of which are coding for the ets DNA binding domain. The last three exons are separated by very small introns (88–387 bp) in contrast to the first intron that is ~5 kb (Figure 2). The intron/exon splice junctions are identical in both genes. The overall nucleotide identity between the human and the mouse gene is 90% within the coding region (Figure 3B and drops to 50–60% within the sequenced introns and the 3′ untranslated regions (not shown).

The deduced amino acid sequence of the mouse gene is 98% homologous to the human gene (Figure 3A), although the mouse protein is three amino acids longer than the human protein (551 *versus* 549 aa). The proteins are identical within the DNA binding domain and all of the seven putative MAP kinase sites can also be found within the mouse sequence at exactly the same positions. Interestingly, six out of the 10 non-conservative mutations can be found within the repressor domain of ERF genes (amino acids 476–529 of the human protein).

Promoter analysis

In order to determine if the previously identified ERF cDNA contained the entire ERF mRNA, we analysed the genomic DNA sequence surrounding the 5′ end of the ERF cDNA for its ability to function as a promoter. Immediately upstream from the area that corresponds to the 5′ end of the cDNA, there is a putative TATA element conserved both in mouse (Figure 2A) and human (Figure 5A). This area has a very high GC content consistent with the promoter of a ubiquitously expressed gene, such as ERF. Indeed, ERF can be

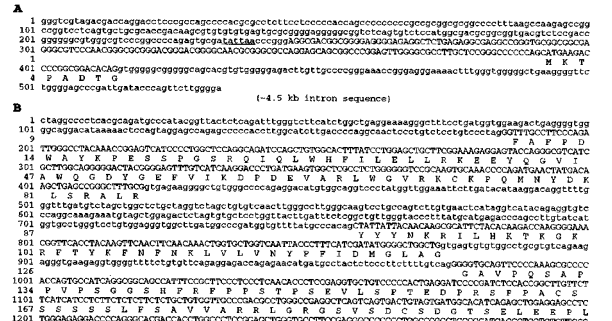

Figure 2 (**A**) Nucleotide sequence of the mouse gene promoter region. The putative TATA box is underlined. Capital letters depict sequences homologous to the human *ERF* cDNA. The predicted amino acid sequence is represented by the single letter code under the corresponding codons. (**B**) Sequence of the 2nd, 3rd and 4th exons of the mouse *Erf* gene. Capital letters as in (**A**). The numbering of the amino acid sequence is continuous between (**A**) and (**B**). (**C**) Sequence of the splice junctions of intron 1 and introns 2 and 3 of the human *ERF* gene. Capital letters as in (**A**)

detected in all cell lines and tissues tested, similar levels, when compared to actin mRNA (Figure 4).

There is a remarkable homology between the mouse and the human gene within the 250 bp region upstream of the putative initiation point. The extent of homology is equivalent to that of the coding region, i.e. ~91% (Figure 3C). In addition to the TATA box, a number of other putative transcription factor binding sites are conserved in sequence and relative position between the two species, including two Sp1 sites, one CREB/ATF site and one ets binding site (EBS). The EBS site within the mouse promoter is actually 13 bp upstream of the corresponding position within the human promoter (Figure 3C).

We tested the ability of a 330 bp fragment of the human gene that harbors the area that corresponds to the 5′ end of the cDNA, to function as a promoter after transfection in HeLa cells. As shown in Figure 5B, this fragment exhibited an orientation-specific promoter function. The promoter strength of this fragment was 50% of the *ETS2* promoter (Mavrothalassitis *et al*., 1990a,b) when tested in the same vector, cells and under the same conditions (data not shown). This is consistent with the relative mRNA levels of ERF and ETS2 in HeLa cells (not shown). Interestingly, when an 80 bp fragment that contains the putative ets binding site and one Sp1 was removed,

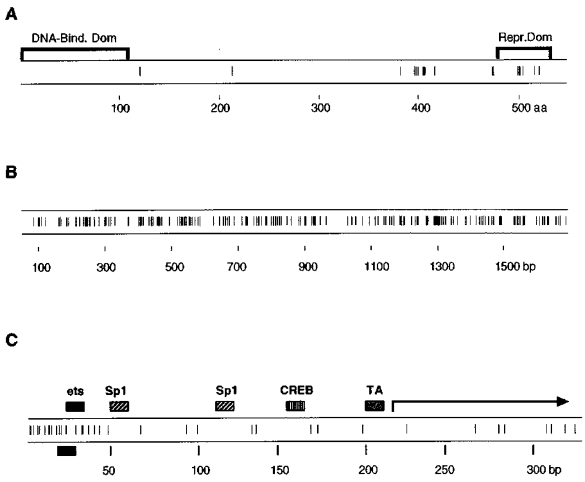

Figure 3 Graphical representation of the homology between the human and mouse proteins (**A**), nucleotide sequence (**B**) of coding regions and promoter sequences (**C**). The graphs were generated by the GapShow program of the GCG analysis package. The vertical lines indicate non-identical positions. The area that corresponds to the DNA-binding and repressor domains are indicated in (**A**). The putative transcription factor recognition motifs, conserved between the human and the mouse promoter sequence, are indicated in (**C**). Note that the position of the putative ets binding site is slightly shifted in the mouse sequence. The arrow in (**C**) indicates the area of identity with the human *ERF* cDNA

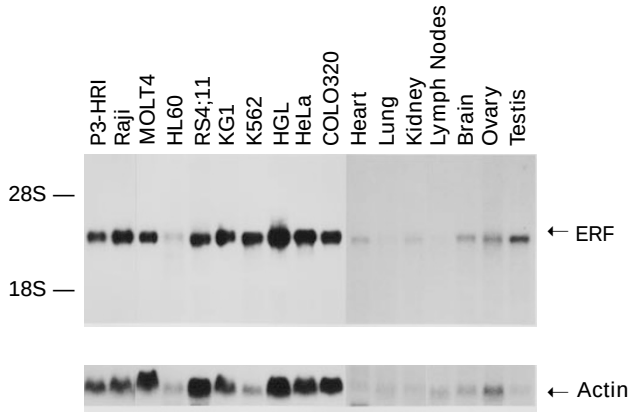

Figure 4 Northern analysis of the *ERF* mRNA levels. The indicated cell line and tissue RNAs were analysed by Northern blot. The ERF cDNA and the *β*-actin cDNA were used as probes, as indicated

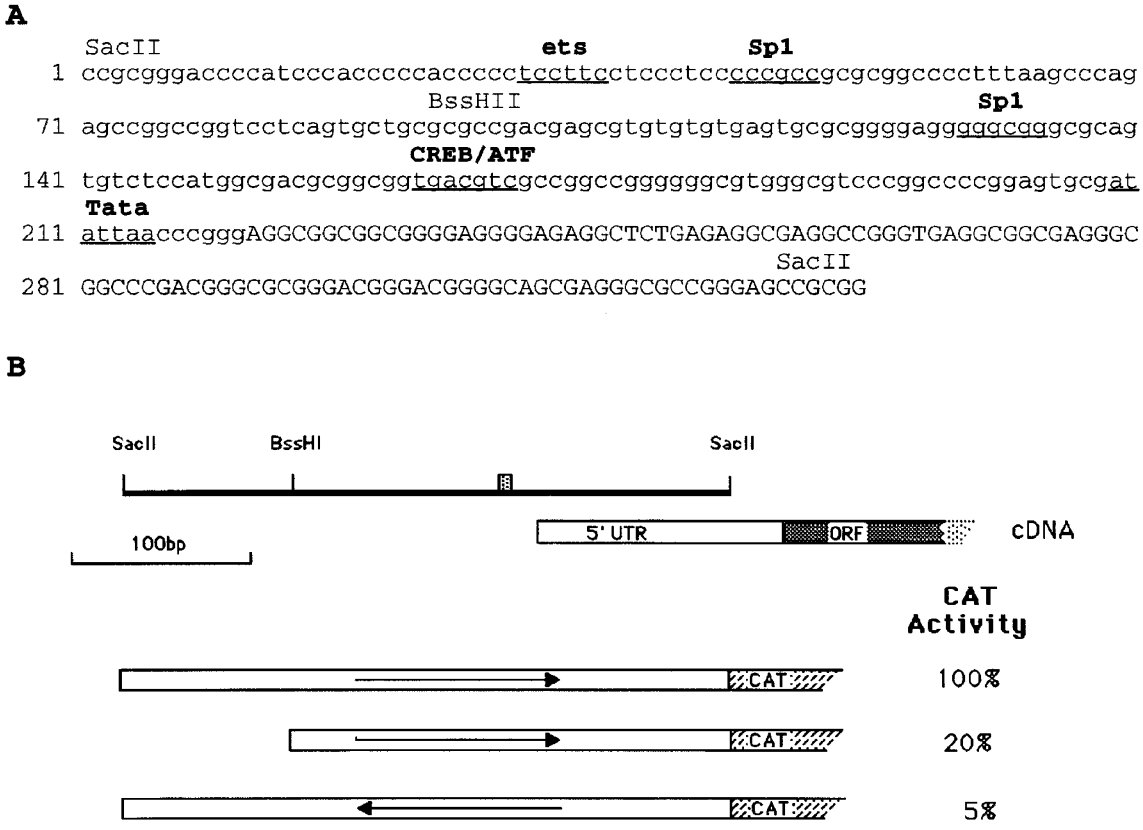

Figure 5 The promoter of the human *ERF* gene. (**A**) Nucleotide sequence of the *ERF* promoter region. The putative transcription factor binding sites are underlined and the putative transcription factor is represented by bold letters. The relevant restriction sites are indicated over their recognition sequence (**B**). A schematic representation of the *ERF* promoter region and the reporter constructs used to determine promoter activity. Promoter strength is expressed as a percentile of the activity of the 332 bp SacII fragment. Striped box indicates the position of the putative TATA box

the promoter activity was dramatically reduced, suggesting that this area is important for the promoter function in this cell type.

Chromosomal localization

The ability of the ERF gene to suppress ets-induced tumorigenesis suggested that it may have some tumor suppressor function. To identify possible human malignancies which may be associated with *ERF* function, we determined its chromosomal position in both human and mouse. Southern analysis with an *ERF*-specific probe of somatic cell hybrid panels (BIOS Corp., New Haven, CT) revealed that *ERF* is localized on human chromosome 19 (data not shown). For the regional assignment of *ERF*, we performed fluorescent *in situ* hybridization (FISH) analysis using both cosmids no. 7 and no. 20 (Figure 1) carrying the human *ERF* gene as a probe. A total of 67 metaphase cells were examined. Twenty-five of these cells revealed paired hybridization signals and an additional 24 cells exhibited single signals at chromosomal region 19q13.1. Consistent background hybridization was not observed at any other specific chromosomal position (Figure 6).

In addition to the mapping of the human gene, we have determined the chromosomal position of *Erf* in mice by linkage analysis. We performed genotypic analysis of about 60 random back-cross progeny mice using a mouse *Erf* probe that can detect a Taq1 polymorphism in *Mus spretus* and C57BL/6 genomic DNA (data not shown). Analyses of the genotypic data, using the mapping service of the UK Human Genome Mapping Program Research Center, have indicated that the mouse *Erf* gene is localized on chromosome 7 close to the Pkcc anchor marker proximal to the centromere (Lod:3.19). This chromosomal region is syntenic to the human chromosomal 19q13 region where the Pkcc marker is also localized and consistent with our mapping data of the human gene.

$(GT)_n$ dinucleotide repeat polymorphism

Polymorphic microsatellite sequences serving as molecular markers are particularly useful tools in human chromosome genetic and physical mapping and, potentially, in genotype-phenotype correlations. To identify these sequences, which are closely linked to human *ERF*, the two overlapping cosmid clones containing the gene, pEW15 no. 20 and pEW15 no. 7 (Figure 1), were investigated by Southern analysis. A 380 bp *Hae*III fragment from pEW15 no. 20 yielded a strong hybridization signal after probing to a synthetic (GT)-19-oligonucleotide. The fragment was partially sequenced (EMBL accession No. X97703) to identify the GT repeat and to provide information for appropriate PCR primers flanking the repeat. Genotyping across the CEPH (Centre d'Etude du Palymorphigme Humain) parental DNAs determined four alleles, i.e. A1 (167 bp), A2 (169 bp), A3 (171 bp) and A4 (173 bp) (data not shown). The observed heterozygosity was 53%. Allele frequencies estimated from 154 chromosomes of 77 unrelated individuals, i.e. parents of the three generation CEPH (Paris) families, were: 0.30, 0.51, 0.17 and 0.02, respectively. Co-dominant segretation was observed in seven large CEPH families tested, ie, no. 1333, no. 1340, no. 1344, no. 66, no. 102, no. 1418 and no. 12, suggesting the stability of the repeat and thus its applicability as an *ERF* linked microsatellite genetic marker.

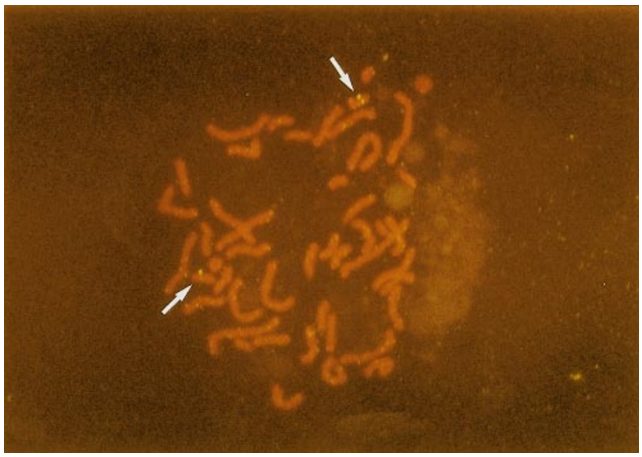

Figure 6 Chromosomal localization of the *ERF* gene. Metaphase chromosome following FISH with the pEW15 no. 7 and no. 20 genomic clones. Arrows indicate specific hybridization at 19q13.1

Discussion

ERF, a new *ets* domain protein (Sgouras *et al*., 1995), is the first mammalian member of this family that exhibits transcriptional repressor activity. This *ERF* product has been shown to be regulated by phosphorylation during cell cycle and mitogenic stimulation via the *ras*/MAPK signaling pathway (Sgouras *et al*., 1995, and unpublished data). To this extent, it is analogous to the only other *ets*-domain protein that has transcriptional repressor function, the *Drosophila* gene, *Yan* (ONeill *et al*., 1994). Furthermore, *ERF* has been shown to suppress *ets*- and *fos*-induced transformation (Sgouras *et al*., 1995) and phosphorylation deficient mutant of ERF can suppress *ras*-induced transformation (to be published elsewhere). Thus, it would appear that *erf* may function as a tumor-suppressor gene, suggesting that part of the *ets* oncogenic phenotype may be associated with the inhibition of *erf* function.

In order to further our understanding of *ERF* function, its association with other *ets* genes and its possible impliction in malignant processes, we have characterized the gene structurally from both human and mouse, determined its chromosomal localization, identified a potentially useful microsatellite polymorphic marker and characterized its promoter region. Structural analysis of the human and mouse genes indicated a high degree of conservation between the two species both at the intron/exon organization and sequence level. This is consistent with our preliminary data indicating that the *erf* gene can be detected by Southern analysis in species throughout evolution from chicken to man (unpublished data). Comparison between the human and mouse gene suggests that the targets of the gene should be identical in the two species. This is apparent from the identity of the DNA-binding domains. The conservation of all the recognizable motifs, including the

MAPK sites and the putative SH3 interaction domains, indicate that the possible regulatory mechanism should also be conserved. Interestingly, a number of mutations can be found in the area previously defined as the repressor domain. Since the human gene can effectively repress transcription in mouse cells (Sgouras *et al*., 1995, and unpublished data), it is unlikely that these differences are a result of diverging co-factors required for repressor function. Thus, it is likely that these amino acid differences provide a more narrow definition of the *erf* repressor domain. However, this hypothesis requires further testing.

The DNA-binding domain of *erf*, as in most of the characterized *ets* genes, is encoded by two exons (exons II and III). However, the intron-exon boundaries of *erf* are distinct from other *ets* genes, indicating an early divergence of *erf* from the other *ets* genes in evolution. This observation is consistent with the positioning of *ERF* in the *ets* gene evolutionary tree. Computer analysis performed by comparison of the *ets*-DNA-binding domains of the known *ets* family members by the PileUp program of the GCG package, positioned *erf* between *Yan* and *PE-1* (data not shown).

In addition to the structural and sequence conservation of *erf* between human and mouse, we also observed a conservation in their respective chromosomal positions. Thus, the human gene was localized in chromosome 19q13.1 and the mouse gene in the syntenic position proximal to the centromere of chromosome 7. Both the human chromosome 19 and the mouse chromosome 7 have been associated with a number of abnormal phenotypes. Specifically, human chromosome 19 has been associated with a number of hematopoietic malignancies that involve translocations or trisomies (Johanson *et al*., 1994; Stark *et al*., 1995). Although the most common translocation, t(14;19)(q32;q13), involves the BCL3 gene (Tanaka *et al*., 1990; Yabumoto *et al*., 1994; Ohno *et al*., 1993), a number of case reports referring to abnormalities in chromosome 19q13 have been published (Paietta *et al*., 1988; Belge *et al*., 1992; Bartnitzke *et al*., 1989). An increasing volume of data implicates *ets* genes in lymphoid malignancies, suggesting a role for *ets* genes in lymphoid development and differentiation. To this extent, it is plausible that *ERF* may be involved in some of the malignancies associated with 19q13 abnormalities. Chromosome 19 abnormalities have also been reported in a number of solid tumors in breast, lung and GI tract. Although solid tumors usually display multiple chromosomal abnormalities, it is possible that *ERF* deregulation may contribute to the transformed phenotype. The possible involvement of ERF in growth regulation suggests that loss or modulation of *ERF* function may contribute to the proliferative aspect of the malignant phenotype. However, extensive analysis is required in order to establish any possible association of *ERF* with these cases. Thus, the microsatellite polymorphic marker that we have identified proximal to the *ERF* gene should facilitate the determination of a possible linkage of ERF with the transforming phenotype.

Another interesting point of the comparison between human and mouse *erf* is the high degree of conservation in their promoter region. The region that is surrounding the area homologous to the 5′ end of the human *ERF* cDNA is 91% identical between the two species and for the first 150 nt upstream of the cDNA end contain no gaps. The degree of structural conservation in this area is equal to that of the coding regions, and it is in clear contrast to other sequenced areas of the two genes as the second and third introns and the 3′ untranslated regions, which have a low degree of similarity (i.e. 50–60%). This region can serve as a promoter in transient transfection assays and contains a number of putative binding sites for transcription factors that are conserved both in sequence and in relative positions, suggesting that the level of *ERF* transcription may be important. Indeed, in most tissues and cell lines tested, ERF mRNA levels exhibit little difference and it is not clear at this point whether these minor observed differences also reflect protein levels or are compensatory for protein turnover among different cell types. We were unable to detect any difference in *ERF* transcription as a function of cell cycle or growth stage (unpublished data) and we could only observe a dramatic decrease in *ERF* mRNA levels in cultures that were entering density crisis and consequently apoptosis (unpublished data). However, under these conditions most of the cellular transcription is terminated and is unknown at this point whether loss of ERF function might be a contributing factor in this process.

It is of interest that a putative *ets*-binding site (EBS), adjacent to an Sp1 site, within the ERF promoter is required for promoter function. Although further experiments are required to determine the contribution of the EBS, by itself and in association with the Sp1 site, in the regulation of ERF transcription, it is suggestive of a possible feedback loop in *ERF* regulation. *ERF* was isolated by its association with the *ETS2* promoter regulatory sequence, and our data indicate that it may itself be regulated by other *ets*-domain proteins, suggesting an additional level of coordinated regulation among *ets* family members.

Materials and methods

Isolation and analysis of erf genes

The human placenta cosmid and the bacteriophage lambda 129SVJ libraries were purchased from Stratagene (Cat. Nos. 951202 and 946309, respectively) and were screened according to the protocols of the company. Duplicate filters were screened with the entire *ERF* cDNA as a probe. Restriction mapping, Southern and Northern blot analysis, subcloning and sequencing, as well as all other molecular techniques were performed according to Sambrook *et al*. (1989). Total RNA from cell lines and tissues was isolated with RNazol according to the manufacturer's specifications. Computer analysis was performed with the University of Wisconsin GCG package.

Cell lines and transfections

HeLa cells were maintained in DMEM supplemented with 10% bovine serum. P3-HR1, Raji, MOLT4, HL60, RS 4;11, KG1, K562, HGL and Colo320 cells were maintained in RPMI with 15% fetal bovine serum. The cells were transfected with the Calcium Phosphate method according to Gorman *et al*. (1982) with 1–3 μg reporter plasmid. One μg pCH110 plasmid (Pharmacia) was used to monitor the transfection variation. All transfections were performed at

least in triplicate with two independent DNA preparations. Determination of CAT activity was performed with a diffusion based assay using ^{14}C-labeled acetyl-CoA (NEN) according to the manufacturer's protocol. DNA fragments to be tested for promoter activity were subcloned at the NotI site of the pUMS P-L vector (Jorcyk *et al*., 1991).

Chromosomal mapping

Fluorescence *in situ* hybridization was performed as described (Tory *et al*., 1992). Briefly, the two pEW15 genomic clones no. 7 and no. 20 were pooled and labeled with biotin-11-dUTP using nick translation. Hybridization was performed at a probe concentration of 30 ng/microliter at 37°C for 16 h followed by washing at 40°C in 50% formamide in 2×SSC. Slides were then incubated in a detection solution containing 5 μg/ml fluorescein isothiocyanate (FITC)-conjugated avidin. Chromosome identification was mediated using QFH banding by simultaneous Hoechst 33258 staining.

Genetic linkage analysis

Genetic linkage analysis was performed with the support and technical advice of UK Human Genome Mapping Programme (HGMP), Resource Center which provides the facility based on an interspecific backcross between c57BL/6 and *Mus spretus* for the genetic mapping of mouse probes on the mouse genome (Genome News, 1995). Genotypic analysis of a panel of about 50 random backcross progeny mice was performed by Southern blots using as a probe a mouse ERF 950 bp KpnI fragment. Earlier, this fragment was found to detect a TaqI polymorphism on *Mus spretus* and C57BL/6 parental polyblots provided by the Resource Center (data not shown). Linkage to chromosome 7 was carried out at the Resource Center by haplotype ordering. Relevant computation of linkage was obtained through the MBx database where mouse, locus, probe and allele data at each chromosome locus for each of 1000 backcross progeny, are stored.

GT-dinucleotide polymorphism

The oligonucleotides CTGAGGGGTTATTCTGTCTC and AGCCAGGTGCAGAGTAATAC were used to determine a possible $(GT)_n$ polymorphism by PCR. The PCR reaction was performed in a volume of 25 ml containing 30 ng of DNA, 35 pmoles of each of the primers, 0.3 pmole of ^{32}P-labeled GT-strand primer, 250 mM of each dATP, dCTP, dGTP and dTTP, 5 mg BSA, 0.5 U Taq polymerase (Minotech), 10 mM Tris HCl pH 8.3, 50 mM KCl and 1.5 mM $MgCl_2$, initial denaturation at 94°C (5 min) was followed by 35 cycles with denaturation at 94°C (45 s), annealing at 57°C (60 s). And extension at 72°C (2 min). The final extension step was for 7 min. Products were resolved on 6% polyacrylamide/urea gels. Allele sizes were estimated by comparison to a M13mp18 sequencing ladder. The most intense band for each allele was used to obtain the allele size. Genomic DNA from CEPH families was provided by GENETHON to NKM, in the context of the EUROGEM project.

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Accession numbers

The nucleotide sequence data reported in this manuscript have been submitted to GenBank and assigned the accession numbers; X97703, U58533, U58534, U58545, U58536, U58537, U58538 and U58539.

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