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Characterization of NERF, a Novel Transcription Factor Related to the Ets Factor ELF-1

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We have cloned the gene for a novel Ets-related transcription factor, new Ets-related factor (NERF), from human spleen, fetal liver, and brain. Comparison of the deduced amino acid sequence of NERF with those of other members of the Ets family reveals that the level of homology to ELF-1, which is involved in the regulation of several T- and B-cell-specific genes, is highest. Homologies are clustered in the putative DNA binding domain in the middle of the protein, a basic domain just upstream of this domain, and several shorter stretches of homology towards the amino terminus. The presence of two predominant NERF transcripts in various fetal and adult human tissues is due to at least three alternative splice products, NERF-1a, NERF-1b, and NERF-2, which differ in their amino termini and their expression in different tissues. Only NERF-2 and ELF-1, and not NERF-1a and NERF-1b, function as transcriptional activators of the *lyn* and *blk* gene promoters, although all isoforms of NERF bind with affinities similar to those of ELF-1 to a variety of Ets binding sites in, among others, the *blk*, *lck*, *lyn*, *mb-1*, and immunoglobulin H genes and are expressed at similar levels. Since NERF and ELF-1 are coexpressed in B and T cells, both might be involved in the regulation of the same genes.

In our search for transcriptional regulators of B-cell differentiation we have observed that the majority of B-cell-specific genes contain *ets*-related enhancer elements in their regulatory regions (4). We, therefore, have focused on characterizing the members of the *ets* transcription factor/oncogene family which are involved in B-cell gene regulation. The *ets* gene family traces its history to oncogenes cloned from retroviruses (89). Since the time of this original cloning, more than 20 cellular homologs which function as transcription factors under physiological conditions and transform cells when aberrantly expressed have been cloned (36, 89). All members of the Ets family share a highly conserved 80- to 90-amino-acid DNA binding domain, the Ets domain. The Ets domain is sufficient to interact specifically with DNA sequences, and because of the conserved DNA binding domain, binding sites for *ets* factors are all very similar, with a core binding motif, A/GGAA/T, and slight differences in flanking nucleotides for different *ets* factors (36, 89). Outside the DNA binding domain very little homology is common to all members of the Ets family. Ets-related proteins can be grouped into subclasses based on additional homologous domains unique for particular members of the Ets family (36, 89). Thus, for example, ERP, SAP-1, and ELK-1 are grouped together because of three homology regions outside the Ets domain which are not found in other members of the Ets family (16, 55).

The role of *ets* factors in human carcinogenesis was recently established when several members of the Ets family were directly implicated in specific chromosomal translocations in different types of cancer. Ewing's sarcoma is characterized by translocations involving either erg, fli-1, or ETV-1, three closely related *ets* factors, and the *EWS* gene (17, 37, 97), whereas in a subset of AML *erg* is translocated to the *FUS* gene (66). Another member of the Ets family, tel, is linked to translocations of chromosome 12 in a variety of different myeloid and B-cell leukemias leading to fusion of *tel* to several different genes including *AML1*, *abl*, and the platelet-derived growth factor receptor (PDGF-R) gene (25, 26, 67). Each fusion protein is associated with a specific type of leukemia; thus, fusion of tel to AML1 leads to pre-B-cell leukemia (25), whereas tel–PDGF-R fusions are associated with chronic myeloid leukemia (26).

Members of the Ets family play an essential role in the transcriptional control of primarily stringently regulated genes such as those involved in tissue specificity, differentiation, and proliferation. Thus, many B- and T-cell-specific genes contain functionally relevant binding sites for *ets* factors, including immunoglobulin (Ig) heavy and light chains; lck; blk; lyn; terminal deoxynucleotidyltransferase (TdT); mb-1; B29; interleukin-2 (IL-2); IL-2 receptor α and β; CD3; CD4; and T-cell receptor α, β, and δ (4, 20, 21, 28, 35, 49, 50, 53, 60, 63, 81, 95). ELF-1 is a member of the Ets family and was originally viewed as a T-cell-specific transcription factor (36, 89) regulating the expression of IL-2, IL-2 receptor α, granulocyte-macrophage colony-stimulating factor, IL-3, CD4, human immunodeficiency virus type 2 (HIV-2), and human T-cell leukemia virus type 1 (HTLV-1) (14, 27, 38, 49, 50, 63, 86, 95). We have recently demonstrated that ELF-1 is highly expressed in B cells, being involved in the regulation of a whole set of genes in those cells, including the IgH, *blk*, *lyn*, TdT, *B29*, *lck*, and *mb-1* genes (4).

A characteristic feature of all *ets* factors is their ability to interact with other classes of transcription factors. Hence, whereas the related *ets* factors SAP-1 and ELK-1 are able to form a ternary complex with the serum response factor, GABP-α forms dimers with GABP-β and Pu.1 interacts with Rb, TFIID, and Pip (36). This characteristic is displayed in the

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structural diversity of different *ets* factors outside of the DNA binding domain. However, the divergent regions are not the only regions involved in protein-protein interactions. Thus, ELF-1 forms a complex with hypophosphorylated retinoblastoma protein Rb via the unique amino terminus (88) and interacts with NF-κB p50, c-rel, c-jun, and HMG I via the DNA binding domain (7, 38), suggesting that interaction of ELF-1 with a variety of transcription factors might be a biologically important feature.

To search for novel members of the *ets* family which might be relevant for B- and T-cell gene regulation, a human cDNA database was searched for sequences homologous to known members of the Ets family. We now report the isolation and characterization of cDNA clones encoding three alternative splice products of a novel member of the *ets* gene family, named new Ets-related factor (NERF), which differ in their amino termini. The NERF gene is most closely related to ELF-1 (81) both in the DNA binding domain and at the amino terminus. We have analyzed the expression pattern of NERF mRNA by Northern (RNA) blot hybridization. We show, furthermore, that the different splice products of NERF bind with high affinity to *ets* sites in various B- and T-cell-specific enhancers and promoters which are also targets for ELF-1 but differ in their transactivation capacities. Since NERF and ELF-1 are coexpressed in B and T cells, both factors may be involved in the regulation of the same genes.

MATERIALS AND METHODS

Cell culture. BASC6C2 (murine pre-B cells), NFS 5.3 (murine late pre-B cells), A-20 (murine mature B cells), EL-4 (murine T cells), Pu5-1.8 (murine monocytes), REC (murine renal epithelial cells), CV-1, COS, and HeLa cells were grown as described previously (53).

Isolation of poly(A)$^+$ mRNA. Poly(A)$^+$ mRNAs were isolated as described by Libermann et al. (52).

Isolation and analysis of cDNA clones encoding a novel *ets*-related protein. To search for novel members of the Ets family, a human cDNA database consisting of approximately 500,000 expressed sequence tags (ESTs) was searched for sequences homologous to the known Ets members by the blastn and tblastn sequence alignment algorithms (5). EST clones were discovered by established EST methods (1, 2). Several ESTs with nucleotide sequence identity to each other were found in the database and were predicted to encode a novel Ets-like protein. These ESTs originated from cDNA clones prepared from several libraries, including human infant brain, IL-1-induced human smooth muscle cell, human microvascular endothelial cell, and human stomach libraries. One 2.5-kb cDNA clone originating from a human infant brain library was selected, sequenced to completion, and chosen for further study.

5′ RACE primer extension. Human spleen and fetal liver cDNA ready for 5′ rapid amplification of cDNA ends (5′ RACE) primer extension was obtained from Clontech, and 5′ RACE PCR amplification was performed according to the manufacturer's instructions with a 5′ anchor primer (AP-1), 5′-CTGGTTCGG CCCACCTCTGAAGGTTCCAGAATCGATAG-3′, and nested primers specific for the 5′ end of the partial NERF cDNA, i.e., N1 (5′-AGAGACAGCCT TTGAATCCACCAGC-3′) and N2 (5′-CTCAGGAGACCCATTGGAAATTG GTG-3′).

5′ RACE PCR amplifications were carried out in a final volume of 50 μl containing 100 ng of 5′ RACE cDNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphosphates, 0.5 μl of *Taq* DNA polymerase (Promega), and 10 pmol of the anchor primer (AP-1) with 10 pmol of either N1 or N2. Reaction mixtures were overlaid with mineral oil and amplified by using a Perkin-Elmer Cetus thermal cycler 480 as follows: 30 cycles of 45 s at 94°C, 45 s at 60°C, and 2 min at 72°C followed by 7 min at 72°C. After two rounds of PCR amplification, 10-μl portions of the reaction products were analyzed on 2% agarose gels. Forty-microliter portions of the relevant amplified DNA fragments were subcloned into the TA cloning vector (obtained from Invitrogen). Colonies of recombinant clones were randomly picked, and minipreparations of plasmid DNA were prepared with a Wizard miniprep kit from Promega. The isolated plasmid DNA was digested with *Eco*RI and analyzed on a 0.8% agarose gel. Double-stranded cDNA inserts of various lengths present in plasmid preparations from several different PCR amplifications were sequenced on an Applied Biosystems automatic DNA sequencer model 373A using the Taq DyeDeoxy Terminator Cycle Sequencing Kit and a combination of specific oligonucleotide primers.

All of the clones sequenced contained NERF-specific cDNAs of various lengths which extended toward the 5′ end. Three alternative splice products of NERF, i.e., NERF-1a, NERF-1b, and NERF-2, which differed in their 5′ end sequences, were identified. The 5′ end sequence of the NERF cDNAs was confirmed by repeating 5′ RACE PCR amplification with primers specific for the 5′ ends of the longest 5′ RACE products obtained in the first two rounds of PCR amplification, i.e., N3 (5′-CGGATGAGCAGATCCAGCTGGTTCG-3′) and N4 (5′-CCTGCTCTAATCTGGCACTTGGAAC-3′). Sequencing of the PCR products confirmed the apparent 5′ ends of NERF-1 and NERF-2.

DNA sequencing. All cDNA clones were sequenced at either the Molecular Biology Core Facility, Dana-Farber Cancer Institute; Human Genome Sciences; or the Beth Israel Hospital DNA sequencing facility by using an Applied Biosystems automatic DNA sequencer model 373A, the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems), and a combination of specific oligonucleotide primers.

DNA and protein sequence analysis. DNA and protein sequences were analyzed and aligned by using the following programs: Mac Vector, LaserGene, DNA Strider 1.2, Gene Jockey, Blast, Seqapp, and Clustal V.

In vitro transcription-translation. Full-length NERF cDNAs encoding the whole open reading frames were inserted into the TA cloning vector (Invitrogen) or Bluescript KS$^+$ (Stratagene) with the T7 or T3 promoter upstream of the initiator methionine. Coupled in vitro transcription-in vitro translation reactions were performed with 1 μg of plasmid DNA by using the TNT reticulocyte lysate kit (Promega) and T7 or T3 RNA polymerase as recommended by the manufacturer in the presence of either [^{35}S]methionine (NEN) or cold methionine. A portion of the labeled in vitro translation products was analyzed on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel as described previously (55).

EMSA. DNA binding reactions and electrophoretic mobility shift assays (EMSAs) were performed as described previously (53, 55). Cell extracts were made from 2×10^5 COS cells after transient transfection for 16 h with 5 μg of NERF and ELF-1 pCI expression vectors. Briefly, cells were harvested, washed with phosphate-buffered saline, and resuspended in 100 μl of luciferase assay lysis buffer containing 1% Triton X-100, 25 mM glycyl glycine buffer (pH 7.8), 15 mM $MgSO_4$, 4 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1% aprotinin (Sigma). Protein concentrations were measured by the Bio-Rad protein assay. Samples of 20 μl containing 2 to 4 μl of in vitro translation product or 2 μl of COS cell extract containing equal amounts of protein were incubated with a solution consisting of 0.1 to 0.2 ng of ^{32}P-labeled double-stranded oligonucleotide probes (5,000 to 20,000 cpm), 10% buffer D (18), 10 mM Tris-Cl (pH 7.5), 66 mM NaCl, 15 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 0.35% Nonidet P-40, 2 μg of bovine serum albumin (Boehringer), and 0.1 μg of poly(dI-dC) (Pharmacia). Samples were incubated in the presence or absence of increasing amounts of competitor oligonucleotides (0.1, 1, and 10 ng) for 15 to 20 min at room temperature and run on 4% polyacrylamide gels (acrylamide-bisacrylamide, 29:1) containing as a buffer 0.5× TGE (1× TGE is 25 mM Tris-HCl [pH 8.5], 190 mM glycine, and 1 mM EDTA).

Oligonucleotides used as probes and for competition studies are listed in Table 1.

Nuclear extracts. Nuclear extracts were prepared according to the method of Dignam et al. (18). All buffers included leupeptin at 0.3 μg/ml, 5 mM phenylmethylsulfonyl fluoride, antipain at 0.3 μg/ml, and aprotinin at 2 μg/ml.

Northern blot analysis. Northern blots containing poly(A)$^+$-selected mRNA derived from different human tissues were obtained from Clontech. Northern blots were hybridized with 2×10^6 cpm of NERF cDNA (labeled with a random primed DNA labeling kit [Boehringer]) per ml at 68°C for 1 h in QuickHyb solution (Stratagene) containing 200 μg of salmon sperm DNA per ml. After washing at 68°C with 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) 0.2% SDS the filters were autoradiographed for 2 days at −70°C using intensifier screens.

RNase protection assay. The RNase protection assay (Ambion RPA II) was performed according to the manufacturer's specifications. In brief, 0.5 to 1 μg of poly(A)$^+$ mRNA or 15 μg of tRNA was hybridized with 10^5 cpm of antisense RNA probe per sample at 45°C overnight. The samples were then treated with RNase for 30 min at 37°C. The protected fragments were ethanol precipitated and separated on a denaturing 5% polyacrylamide gel. Radioactive RNA markers (Ambion) were synthesized, generating fragments of 100, 200, 300, 400, and 500 nucleotides in length.

For the NERF antisense probe, a PCR product corresponding to a fragment of the mouse analog of NERF-1b, from nucleotide 30 to 317 (see Fig. 1), including 25 nucleotides of the NERF-1b alternative splice region, was subcloned. The total length of the NERF probe is 416 nucleotides. The expected sizes of protected RNA fragments for NERF-1a and -1b are 262 and 287 nucleotides, respectively. RNA transcripts of NERF-2 would be partially protected by this antisense RNA probe, from nucleotide 178 on, since nucleotides 30 to 177 are NERF-1a and -1b specific. The expected sizes for the NERF-2a and -2b protected fragments are 139 and 114 nucleotides. The Elf-1 probe corresponds to a 195-nucleotide sequence in the coding region of Elf-1 from amino acid 149 to 214. The total length of the Elf-1 RNA probe is 262 nucleotides. Finally, the mouse β-actin probe (Ambion) is a 250-nucleotide fragment. The relative levels of expression of the different RNA species were quantitated with a Bio-Rad (Richmond, Calif.) phosphor imager. Numbers were adjusted relative to the level of β-actin expression for each RNA. To estimate the relative number of RNA molecules expressed for each RNA species, adjusted phosphor imager numbers

TABLE 1. Oligonucleotides used as probes and for competition studies

Oligonucleotide	Sequence
Murine *blk* promoter WT[a]	5′-TCGAGTCTCCAGGAAGTATTTTTCAGAC-3′
	3′-CAGAGGTCCTTCATAAAAAGTCTGTCGA-5′
Murine *blk* promoter mutant	5′-TCGAGTCTCCACCAAGTATTTTTCAGAC-3′
	3′-CAGAGGTGGTTCATAAAAAGTCTGTCGA-5′
E74 WT	5′-TCGAGTAACCGGAAGTAACTCAG-3′
	3′-CATTGGCCTTCATTGAGTCAGCT-5′
IgH enhancer π WT site	5′-TCGACTGGCAGGAAGCAGGTCATGC-3′
	3′-GACCGTCCTTCGTCCAGTACGAGCT-5′
lck promoter WT	5′-TCGAGGTGGCAGGAAGCTTGG-3′
	3′-CCACCGTCCTTCGAACCAGCT-5′
HSV ICP4 WT[b]	5′-TCGAGCGGAACGGAAGCGGAAACCG-3′
	3′-CGCCTTGCCTTCGCCTTTGGCAGCT-5′
Polyomavirus PEA3 WT	5′-TCGAGCAGGAAGTGACG-3′
	3′-CGTCCTTCACTGCAGCT-5′
MSV LTR WT[c]	5′-TCGAGAGCGGAAGCGCGCG-3′
	3′-CTCGCCTTCGCGCGCAGCT-5′
HIV-2 LTR WT	5′-TCGAGTTAAAGACAGGAACAGCTATG-3′
	3′-CAATTTCTGTCCTTGTCGATACAGCT-5′
HTLV-1 LTR WT	5′-TCGAGGGGAGGAAATGGGTG-3′
	3′-CCCCTCCTTTACCCACAGCT-5′
Stromelysin promoter WT	5′-TCGAGCAGGAAGCATTTCCTGG-3′
	3′-CGTCCTTCGTAAAGGACCAGCT-5′
Urokinase promoter WT	5′-TCGAGTCCAGGAGGAAATGAAGTCAG-3′
	3′-CAGGTCCTCCTTTACTTCAGTCAGCT-5′
T-cell receptor α enhancer Tα2 WT	5′-TCCCGCAGAAGCCACATCCTCTG-3′
	3′-AGGGCGTCTTCGGTGTAGGAGAC-5′
IL-2 NF-AT WT	5′-TCGAGAAAGGAGGAAAAACTG-3′
	3′-CTTTCCTCCTTTTTGACAGCT-5′
IL-2 promoter IL-2B site WT	5′-TCGAGAAGAGGAAAAATGAAG-3′
	3′-CTTCTCCTTTTTACTTCAGCT-5′
fos SRE WT	5′-TCGAGCTTACACAGGATGTCCATATTAGGACATCTG-3′
	3′-CGAATGTGTCCTACAGGTATAATCCTGTAGACAGCT-5′
vpreB promoter WT	5′-TCGAGGGAGGAAGCACCG-3′
	3′-CCCTCCTTCGTGGCAGCT-5′
Ig(κ) 3′ enhancer WT	5′-TCGAGCTTTGAGGAACTGAAAACAG-3′
	3′-CGAAACTCCTTGACTTTTGTCAGCT-5′
MHC class II promoter WT[d]	5′-TCGAGAGTGAGGAACCAATCAG-3′
	3′-CTCACTCCTTGGTTAGTCAGCT-5′
Murine IgH μB WT site	5′-TCGAGCTATTTGGGGAAGGGAAAATAAAAC-3′
	3′-CGATAAACCCCTTCCCTTTTATTTTGTCGA-5′
mb-1 promoter WT	5′-TCGAGAACAGGAAGTGAGGCGGC-3′
	3′-CTTGTCCTTCACTCCGCCGTCGA-5′
Murine *B29* promoter WT	5′-TCGAGCATGGCAGGAAGGGGCCTAC-3′
	3′-CGTACCGTCCTTCCCCGGATGTCGA-5′
Human TdT promoter WT	5′-TCGAGTTTGGCAGGAAGCTGTTGC-3′
	3′-CAAACCGTCCTTCGACAACGTCGA-5′
Human *lyn* promoter WT	5′-TCGAGCACCAGGAAGTAGCTGGGAC-3′
	3′-CGTGGTCCTTCATCGACCCTGTCGA-5′
Human *lyn* promoter mutant	5′-TCGAGCACCATCAAGTAGCTGGGAC-3′
	3′-CGTGGTAGTTCATCGACCCTGTCGA-5′
Murine CD40 promoter WT	5′-TCGAGCCCAGGAAGAGGGCGGGC-3′
	3′-CGGGTCCTTCTCCCGCCCGTCGA-5′
Murine *junB* promoter WT	5′-TCGAGCTGTCAGGAAGCGCGTGTC-3′
	3′-CGACAGTCCTTCGCGCACAGTCGA-5′
Murine IL-10 Pu.1	5′-TCGAGCTGTAATGCAGAAGTTCATTC-3′
	3′-CGACATTACGTCTTCAAGTAAGTCGA-5′
Control	5′-TCGAGCCCTGGTGTGGTAACCCTCC-3′
	3′-CGGGACCACACCATTGGGAGGTCGA-5′

[a] WT, wild type.
[b] HSV, herpes simplex virus.
[c] MSV, murine sarcoma virus.
[d] MHC, major histocompatibility complex.

were divided by the size of the protected fragment, since equal moles of a smaller fragment would contain less radioactivity than a larger fragment.

Expression vector and luciferase reporter gene constructs. A blunted *Xba*I-*Sal*I fragment of the Δ-56-c-*fos*-CAT plasmid as previously described by Gilman et al. (23) containing the c-*fos* minimal promoter region from position −56 to position +107 was inserted into the blunted *Hin*dIII site upstream of the luciferase gene in the pGL3 vector (Promega).

Synthetic wild-type and mutant *lyn* and *blk* promoter *ets* site oligonucleotides as described above containing *Sal*I and *Xho*I ends were inserted as dimers into the *Sal*I site of the Δ56-c-*fos*-pGL3 plasmid.

A human *lyn* promoter fragment was cloned from human genomic DNA (4). A murine *blk* promoter construct containing the *blk* promoter from position −191 through position +136 was kindly provided by Patty Zwollo. The *Kpn*I-*Xho*I *lyn* promoter fragment and the blunted *Pst*I-*Stu*I *blk* promoter fragment were inserted into the *Kpn*I-*Xho*I and *Sma*I sites, respectively, upstream of the luciferase gene in the pGL3 vector (Promega).

A *Bam*HI fragment containing the full-length NERF-1a and NERF-1b cDNAs was blunted and inserted into the *Sma*I site of the pCI (Promega) eukaryotic expression vector downstream of the cytomegalovirus (CMV) promoter. A *Not*I fragment containing the full-length NERF-2 cDNA was inserted into the *Not*I site of the pCI (Promega) eukaryotic expression vector downstream of the CMV promoter. A *Kpn*I-*Xba*I fragment containing the full-length ELF-1 cDNA was inserted into the *Kpn*I-*Xba*I sites of the pCI (Promega) eukaryotic expression vector downstream of the CMV promoter.

DNA transfection assays. Cotransfections of 3×10^5 HeLa cells were carried out with 3.5 μg of reporter gene construct DNA and 1.5 μg of expression vector DNA by using 12.5 μl of Lipofectamine (Gibco-BRL). Cells were washed with serum-free Dulbecco modified Eagle medium (DMEM). A total of 1.6 ml of serum-free DMEM was added per well. Liposomes were incubated with the DNA in 200 μl of serum-free DMEM for 15 min at room temperature and then with the cells for 4 h at 37°C. Two milliliters of DMEM containing 20% fetal calf serum was added, and the cells were harvested 16 h after transfection and assayed for luciferase activity as described previously (65). Transfections for every construct were performed independently in triplicate and repeated two to five times with at least two different plasmid preparations with similar results. Cotransfection of a second plasmid for determination of transfection efficiency was omitted because potential artifacts with this technique have been reported (22) and because many commonly used viral promoters contain potential binding sites for *ets* factors. The protein concentration was measured with a kit from Bio-Rad and normalized for all samples in each individual experiment.

Nucleotide sequence accession numbers. The GenBank nucleotide sequence accession numbers for the reported sequences are U43188 and U43189.

RESULTS

Isolation and characterization of three alternative splice products of the human *ets*-related cDNA, NERF. To search for novel members of the Ets family, a human cDNA database was searched for sequences homologous to the Ets domain. Several ESTs with nucleotide sequence identity to each other were found in the database and were predicted to encode a novel Ets-like protein. These ESTs originated from cDNA clones prepared from several libraries, including human infant brain, IL-1-induced human smooth muscle cell, human microvascular endothelial cell, and human stomach libraries. One 2.5-kb cDNA clone originating from a human infant brain library was chosen for further study. We describe here the isolation and characterization of full-length cDNA clones for this new member of the Ets family which we have named NERF.

Sequence analysis of the original partial cDNA clone revealed homology to the conserved DNA binding domain of the *ets* gene family, with the levels of homology to ELF-1 (50) and E74 (11), a subgroup of the Ets family, being highest (see Fig. 2 and 3). The cDNA clone contained the poly(A) tail and an open reading frame up to the 5′ end of this clone, suggesting that part of the 5′ end was missing.

To determine the 5′ end of NERF, we performed the 5′ RACE method as described in Materials and Methods, using primer N1 close to the 5′ end of our cDNA for priming and primer N2 further 5′ of primer N1 together with an anchor primer, AP1, for PCR amplification. 5′ RACE-ready human spleen and fetal liver cDNAs were obtained from Clontech, and 5′ RACE PCR amplification was performed with the 5′ anchor primer and the nested NERF-specific primers as described in Materials and Methods.

Both strands of the full-length NERF cDNAs were sequenced entirely by double-stranded dideoxy sequencing using T7 and T3 polymerase sequencing primers and NERF-specific primers based on partial DNA sequencing (Fig. 1). All of the 5′ RACE clones sequenced contained NERF-specific cDNAs of various lengths which extended toward the 5′ end. Three alternative splice products of NERF, i.e., NERF-1a, NERF-1b, and NERF-2, which differed in their 5′ end sequences, were identified. The 5′ end sequences of the NERF cDNAs were confirmed by repeating 5′ RACE PCR amplification with primers specific for the 5′ ends of the longest 5′ RACE products obtained in the first two rounds of PCR amplification. That all NERF splice products contain the same 3′ end and are indeed alternative splice forms was confirmed by PCR amplification and sequencing of full-length cDNAs encoding all three splice products. The length of the NERF-1a full-length cDNA is 2,975 bp, that of NERF-1b is 3,011 bp, and that of NERF-2 is 3,240 bp (Fig. 1). These data correlate well with the estimated sizes of the two mRNA species detected by Northern blot analysis (see Fig. 4).

Predicted amino acid sequence of NERF. Sequence analysis of the NERF-1a cDNA revealed a 1,563-nucleotide open reading frame encoding a 521-amino-acid protein with a predicted molecular mass of 56.1 kDa, starting with an ATG at position 122 and terminating with a TAA at position 1685 (Fig. 1). Sequence analysis of the NERF-1b cDNA revealed a 1,599-nucleotide open reading frame encoding a 533-amino-acid protein with a predicted molecular mass of 57.4 kDa, starting with the same ATG as NERF-1a at position 122 and terminating with a TAA at position 1721 (Fig. 1). NERF-1b contains an in-frame insertion of an apparent alternative exon of 36 nucleotides into the 5′ part of NERF-1a. Sequence analysis of the NERF-2 cDNA revealed a 1,743-nucleotide open reading frame encoding a 581-amino-acid protein with a predicted molecular mass of 62.7 kDa, starting with an ATG at position 207 and terminating with a TAA at position 1950 (Fig. 1). The 5′ end of NERF-2 including the 5′ untranslated region is divergent from NERF-1a and NERF-1b because of alternative 5′ exons indicating the presence of two different transcription start sites possibly derived from a proximal and a distal promoter. The ATG initiator codons partially conform to the consensus eukaryotic translation initiation sequence (44) with a G at position +4, a C at position −1, and an A at position −3 for NERF-1a and NERF-1b and a C at position −2 and an A at position −3 for NERF-2. There are several reasons to believe that these ATGs are the translation initiation codons. No additional ATG is found in frame, and in-frame termination codons are found 30 bp upstream of the ATG for both NERF-1a, NERF-1b, and NERF-2. In addition, deletion of all the sequences upstream of the ATG does not alter the size of the protein product, when translated in vitro (62). However, we cannot exclude the possibility that translation of NERF starts at a codon different from ATG. Even though the in-frame insertion of 36 bp in NERF-1b occurs at a position common to both NERF-1 and NERF-2, we have not observed this insertion in any NERF-2 cDNA so far. However, we have evidence from RNase protection assays and reverse transcription PCR that NERF-2 (NERF-2b) mRNA containing the NERF-1b-specific exon exists as well.

The NERF cDNAs contain a long poly(A) tract starting at position 2957 for NERF-1a, position 2993 for NERF-1b, and position 3223 for NERF-2 (Fig. 1) which is preceded by a classical polyadenylation site (Fig. 1) at an appropriate distance. Three ATTTA motifs (Fig. 1), thought to be involved in rapid mRNA turnover (3, 74, 78), are found in the 3′ untranslated region, suggesting low-level stability of NERF mRNAs.

A hydropathicity plot of the predicted amino acid sequences of the different splice products of NERF reveals a primarily hydrophilic amino-terminal half and a slightly hydrophobic carboxy-terminal half. The deduced amino acid sequences of

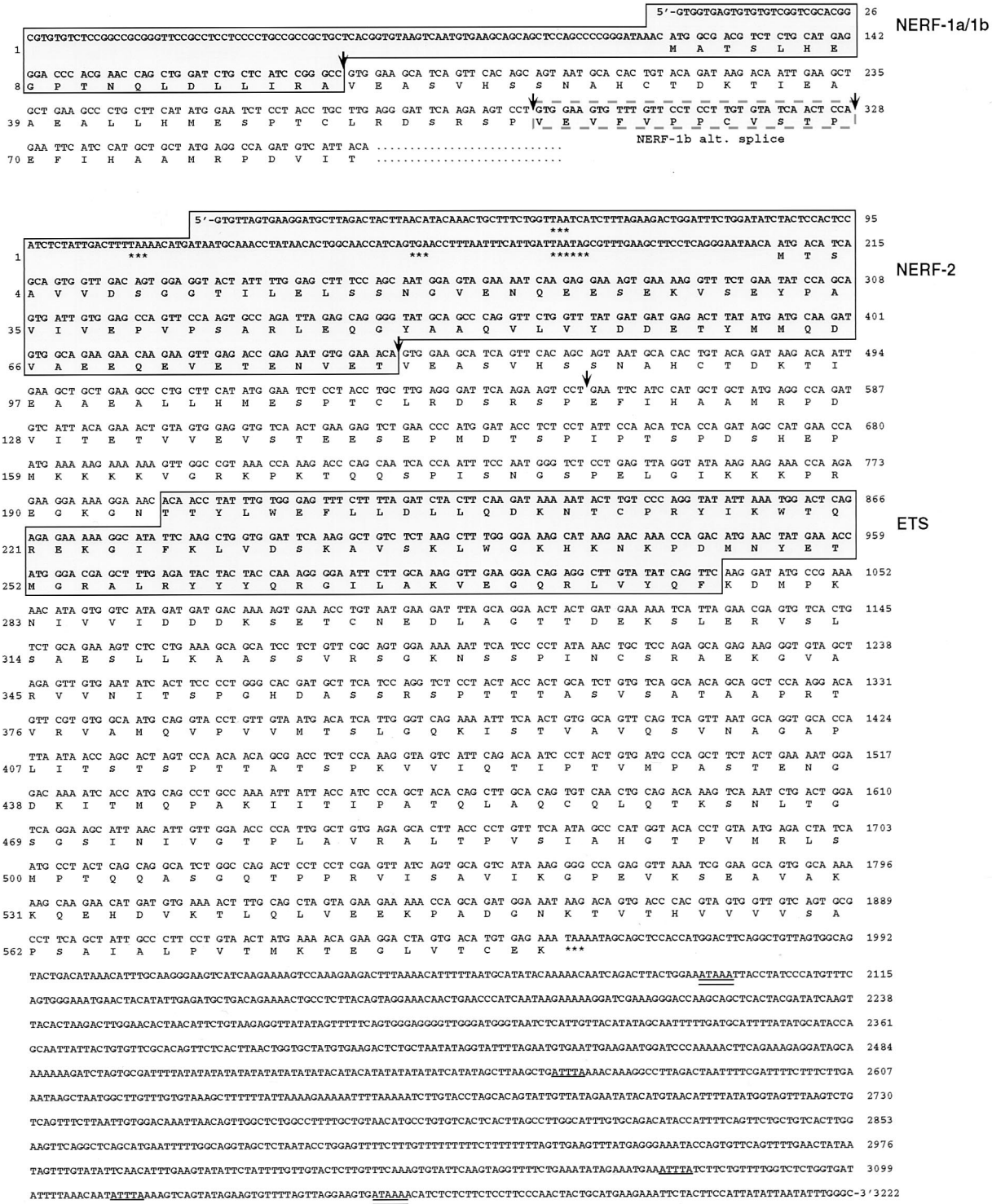


FIG. 1. Complete nucleotide sequences and predicted amino acid sequences of NERF-1a, NERF-1b, and NERF-2. The nucleotide sequences of human NERF-1a, NERF-1b, and NERF-2, with the deduced amino acid sequences (one-letter code) of the major open reading frames, are shown. Nucleotides are numbered on the right; amino acids are numbered on the left. The full-length sequence of NERF-2 is shown in the lower part. The amino-terminal alternatively spliced exons of NERF-1a and NERF-1b are shown in the upper part. The alternative 36-bp exon of NERF-1b inserted into the amino terminus of NERF-1a is boxed by a dashed line and shaded. The NERF-1a and -1b (NERF-1a/1b)- and NERF-2-specific alternative exons and the Ets domain are boxed and shaded and marked on the right side. The arrows indicate the alternative splice sites. The termination codons in frame with the reading frame upstream and downstream are indicated by asterisks. The putative polyadenylation sequence, AATAAA, close to the polyadenylated 3′ end of the mRNA is double underlined. The ATTTA motifs involved in mRNA turnover are underlined.

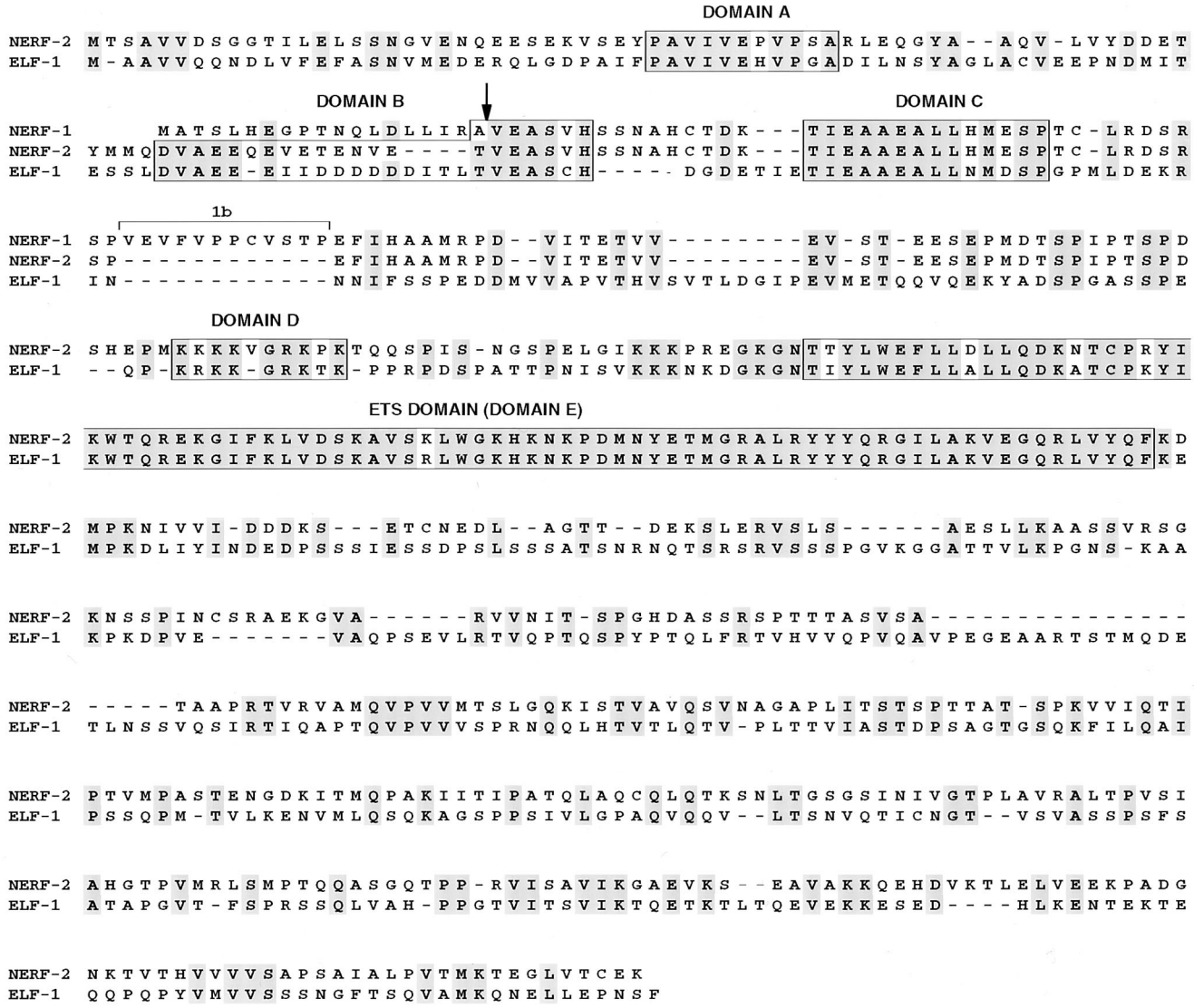


FIG. 2. Comparison of the amino acid sequence of NERF with that of ELF-1. The optimal alignment of the predicted amino acid sequences of NERF-1a, NERF-1b, NERF-2, and ELF-1 (50), obtained by using the Blast, Seqapp, and Clustal programs, is shown. The alternative 12-amino-acid exon of NERF-1b is indicated as an insert in NERF-1a. An arrow indicates the amino acid at which NERF-1 and NERF-2 diverge because of alternative splicing. The five major homology regions, i.e., domains A, B, C, D, and E (the Ets domain), are boxed. Shaded amino acids denote amino acid identity with NERF.

NERF-1a, NERF-1b, and NERF-2 predict proteins rich in alanine (8%), glutamic acid (8%), serine (10%), valine (9 to 10%), threonine (9 to 10%), and lysine (7 to 8%). The amino terminus of NERF, extending from amino acid 33 down to amino acid 85 in NERF-1a and NERF-1b and from amino acid 21 to amino acid 145 in NERF-2, is characterized by a high abundance of aspartic acid and glutamic acid residues (>20%) (Fig. 2). This acidic domain is followed by a 35-amino-acid basic domain and the 85-amino-acid putative DNA binding domain (Ets domain), which is also basic. Downstream of the DNA binding domain is a 35-amino-acid acidic stretch followed by a serine-rich domain. The carboxy-terminal 220 amino acids are high in threonine, valine, glutamine, and alanine, with an intermittent region high in isoleucine. The amino-terminal half contains most of the basic and acidic amino acids. Acidic domains have been observed in a variety of transcription factors and have been implicated previously in transactivation (32, 42, 79). Several potential phosphorylation sites for protein kinase C, casein kinase II, cdc2 kinase, and tyrosine kinase are present in NERF (8, 46, 57, 58, 80). The predicted NERF-1a and NERF-2 protein sequences reveal, furthermore, 15 potential MAP kinase phosphorylation sites (S/TP), one of them containing the optimal PX(S/T)P sequence (6, 34), as well as potential phosphorylation sites for jun kinase and p38 kinase (33, 85). The predicted NERF-1b protein sequence reveals an additional potential MAP kinase or jun kinase phosphorylation site in the alternative exon. Several of the putative kinase phosphorylation sites are present in ELF-1 as well. The importance of MAP kinase phosphorylation sites for the biological function of Ets-related factors has recently been demonstrated (56, 64), suggesting that at least some of these putative sites might be functionally relevant for NERF as well.

Sequence comparison of NERF with other members of the Ets family. Comparison of the deduced amino acid sequence of NERF with those of other members of the Ets family re-

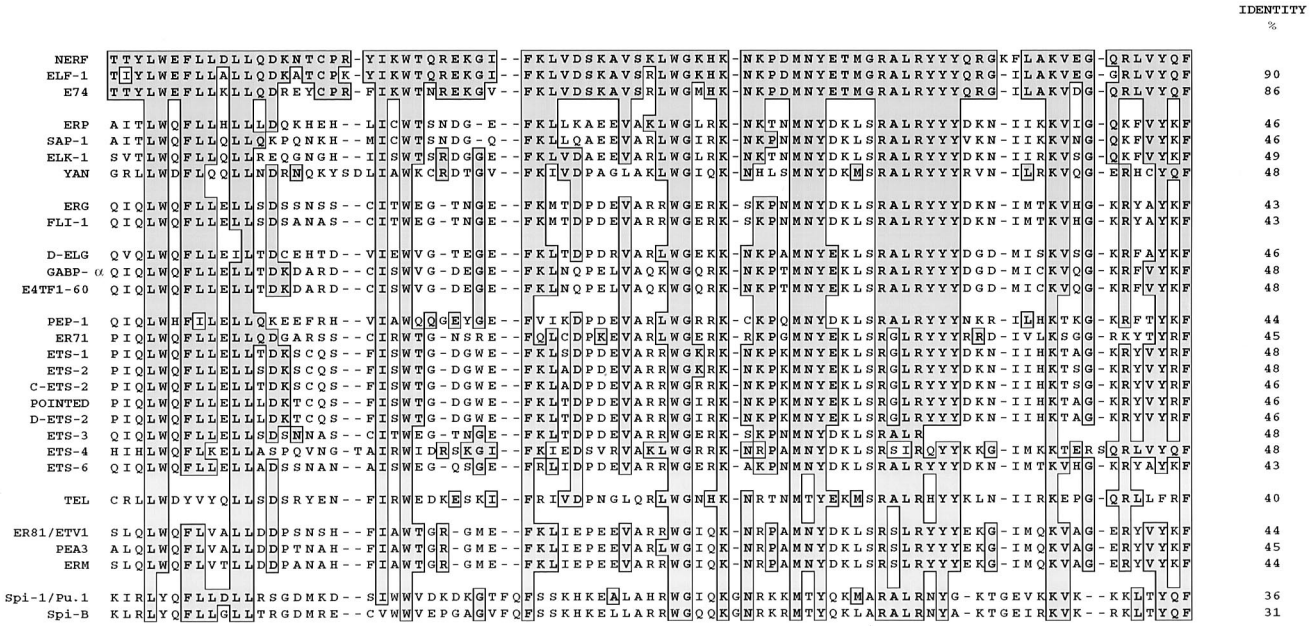


FIG. 3. Comparison of the Ets domain of NERF with all known members of the *ets* gene family. Percent identity with NERF is indicated on the right. Shaded amino acids denote amino acid identity with NERF. Gaps have been introduced to optimize alignment. The proteins examined are indicated on the left and include ELF-1 (50), E74 (11), ERP (55), SAP-1 (16), ELK-1 (72), yan (47), ERG (77), FLI-1 (69), D-ELG (71), GABP-α (48), E4TF1-60 (92), PEP-1 (41), ER71 (10), Ets-1 (12, 76, 93), Ets-2 (93), C-Ets-2 (9), pointed (40), D-Ets-2 (70), Ets-3 (13), Ets-4 (13), Ets-6 (13), tel (26), ETV-1/ER81 (10, 37), PEA3 (96), ERM (59), Spi-1/Pu.1 (43), and Spi-B (73).

vealed that the level of homology to ELF-1 is highest (Fig. 2 and 3). Homologies are clustered in several primary regions, i.e., the putative DNA binding domain E (Ets domain) which extends over approximately 85 amino acids in the middle of the gene, a smaller basic domain (domain D) just upstream of the Ets domain, and several shorter stretches of homology further towards the amino terminus (domains B and C). The NERF-2 gene product contains additional short stretches of homology to ELF-1 at the amino terminus (domain A and part of domain B) which are not present in NERF-1a and NERF-1b. The region towards the carboxy terminus of NERF shows only limited homology to ELF-1. Overall, the amino acid homology of NERF-2 with ELF-1 is 44%.

Alignment of the Ets domain of NERF with those of other members of the Ets family reveals that the levels of homology to ELF-1 (90%) and to a lesser extent E74 (86%) are highest (Fig. 3). Sequence identity to most other members of the Ets family is ~45 to 50%. NERF is least related to Pu.1/Spi-1 (36%) (43) and Spi-B (31%) (73). Homology to other members of the Ets family is restricted to the Ets domain, which is involved in sequence-specific DNA binding (Fig. 3). Interestingly, NERF contains a short stretch of homology to Yan (64) which includes a consensus MAP kinase phosphorylation site thought to be important for Yan function (data not shown).

Expression pattern of NERF in human tissues. To determine the expression pattern of NERF and the size of the NERF transcripts, poly(A)$^+$ mRNAs derived from various human tissues were analyzed by Northern blot hybridization using NERF cDNA as a probe (Fig. 4). To control for RNA quality and quantity, we rehybridized the Northern blots with a glyceraldehyde-3-phosphate dehydrogenase probe.

The results indicate the presence of two predominant NERF transcripts of approximately 2.9 and 3.2 kb (Fig. 4). Further, it was found that the NERF gene is expressed in all tissues tested with significant variation in abundance. Among human fetal tissues, heart, lung, liver, and kidney expressed the largest amounts of NERF (Fig. 4), whereas low levels of NERF mRNA were observed in brain. The 2.9- and 3.2-kb transcripts were expressed in most tissues in approximately equivalent

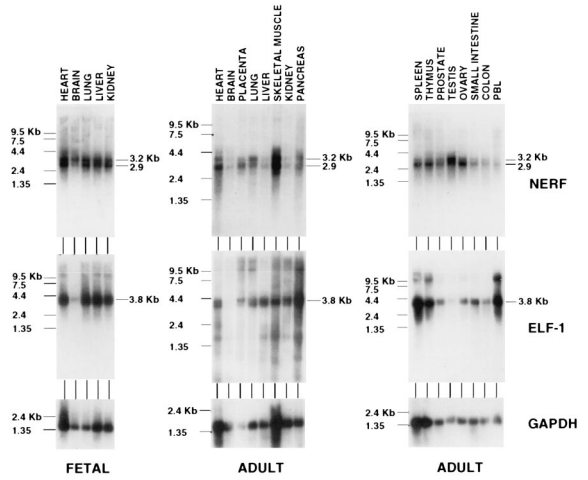


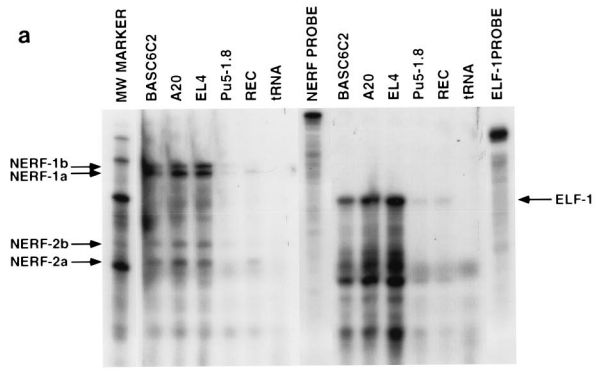
FIG. 4. Expression of NERF and ELF-1 in different human fetal and adult tissues. Northern blot analysis of poly(A)$^+$ mRNA from human fetal tissues (left panel) including heart, brain, lung, liver, and kidney and from adult tissues (center and right panels) including heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and blood (peripheral blood leukocytes [PBL]) was performed. The blot was sequentially probed with a NERF (upper panels), ELF-1 (middle panels), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (lower panels) under stringent conditions as described in Materials and Methods. Numbers on the right indicate sizes of major mRNA bands. The sizes of molecular weight markers are indicated on the left.

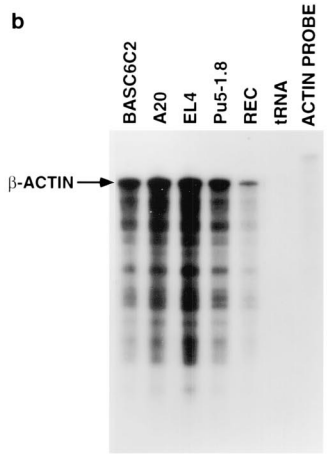
amounts, except in brain, in which the 3.2-kb transcript was predominant. Among adult tissues, heart, placenta, lung, skeletal muscle, spleen, thymus, testis, and ovary expressed the highest levels of NERF (Fig. 4). Moderate levels of NERF transcripts were found in prostate, small intestine, kidney, liver, and pancreas, whereas only low levels of NERF mRNA were expressed in colon, brain, and peripheral blood lymphocytes. The 2.9- and 3.2-kb transcripts were expressed in approximately equivalent amounts only in heart, lung, liver, and skeletal muscle. The 3.2-kb transcript was predominant in testis and brain, whereas the 2.9-kb transcript was more abundant in ovary, spleen, thymus, prostate, small intestine, colon, pancreas, and placenta.

The results suggest that NERF is expressed to various degrees in many tissues. NERF-1a and NERF-1b cDNAs appear to be derived from the 2.9-kb transcript, whereas NERF-2 cDNA is derived from the 3.2-kb transcript. The different transcripts appear to be due to alternative splicing as revealed also by the cloning of the different transcripts. Because the 5′ end of NERF-1 is different from NERF-2, we have to assume that these transcripts are initiated through different promoter regions. Since the transcripts appear to be differentially expressed in different tissues, it is possible that the promoter for NERF-1 is regulated in a manner different from that of the NERF-2 promoter.

Since NERF is highly related to ELF-1, we were interested in knowing where ELF-1 is expressed. Northern blot hybridization analysis was performed with the same blots used for NERF (Fig. 4). ELF-1 is expressed as a main transcript of ~3.8 kb. In fetal tissues ELF-1 expression coincided with NERF expression, with levels being high in heart, lung, liver and kidney but low in brain. For adult tissues the highest levels of ELF-1 were found in pancreas, spleen, thymus, and peripheral blood leukocytes. Moderate amounts of ELF-1 are expressed in heart, placenta, lung, liver, skeletal muscle, kidney, prostate, ovary, small intestine, and colon, whereas brain and testis express very little ELF-1. Thus, the expression patterns of ELF-1 and NERF appear to be slightly different, with ELF-1 expression levels especially high in the immune system and in pancreas and NERF expression levels high in muscular tissues and reproductive organs. Hence, ELF-1 and NERF are coexpressed in various tissues, including spleen and thymus.

To determine in more detail the relative expression levels of the different NERF splice products and ELF-1 in B and T cells, we performed RNase protection assays (Fig. 5). The NERF probe was derived from the NERF-1b cDNA encoding the NERF-1b alternative exon as well as part of the 5′ sequence unique to NERF-1a and -1b. This probe enabled us to detect different-sized protected fragments encoding NERF-1a (262 bp), NERF-1b (287 bp), NERF-2a (114 bp), and NERF-2b (139 bp) (Fig. 5a; see also Materials and Methods). NERF-2a is identical to NERF-2. NERF-2b had not been isolated as a cDNA and is equivalent to the NERF-2 cDNA containing the insertion of the NERF-1b-specific exon. All four NERF splice products as well as ELF-1 (195 bp) were expressed in murine BASC6C2 pre-B cells, A20 mature B cells, EL4 T cells, and Pu5-1.8 monocytic cells. Pu-5-1.8 cells expressed much less NERF- and ELF-1-specific RNAs than did the B- and T-cell lines (Fig. 5). Renal tubular epithelial cells expressed levels of NERF-2a similar to those expressed by the B- and T-cell lines, whereas the level of NERF-1a was three to four times lower than those in the B- and T-cell lines and NERF-1b as well as NERF-2b was not detected. The B- and T-cell lines expressed similar amounts of each NERF splice product. The total amount of NERF RNA expressed was approximately equal to





c

	BASC6C2	A20	EL4	Pu5-1.8	REC
NERF-1b	20	15	14	2	0
NERF-1a	21	20	16	2	6
NERF-2b	22	21	15	2	0
NERF-2a	34	35	18	5	27
ELF-1	72	111	149	11	55

FIG. 5. Expression of different splice forms of NERF and ELF-1 in various cell types. RNase protection assays of poly(A)$^+$ mRNA from BASC6C2 pre-B cells, A-20 mature B cells, EL-4 T cells, Pu5-1.8 monocytic cells, and REC renal tubular epithelial cells were performed with either a NERF or an ELF-1 (a) or a β-actin (b) antisense probe as described in Materials and Methods. RNase protection using tRNA served as a control. The unprotected probes are marked as NERF probe, ELF-1 probe, and actin probe. The molecular weight (MW) markers as indicated on the left are 100, 200, 300, and 400 bp. (c) Relative molar expression levels of different NERF splice forms and ELF-1 in the different cell types. Shown are relative molar equivalents of the different RNA species expressed in different cell types after adjustment for β-actin levels.

the amount of ELF-1 RNA in the B-cell lines. These results suggest that the expression of different NERF splice products is differentially regulated in different cell types and that all splice forms of NERF are expressed in B and T cells at similar levels. Both NERF and ELF-1 are coexpressed in B and T cells, indicating that NERF and ELF-1 may regulate the same target genes.

NERF and ELF-1 bind with similar affinities to functionally important *ets*-related binding sites in a variety of genes. To determine whether NERF can bind in a sequence-specific manner to DNA, NERF-1a, NERF-1b, and NERF-2 as well as

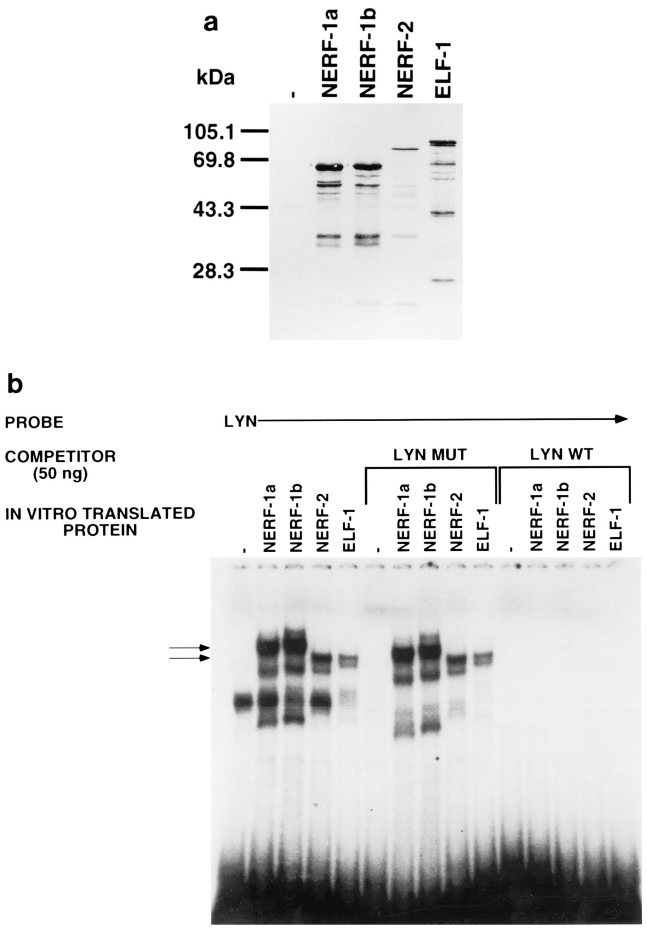


FIG. 6. DNA binding of NERF-1a, NERF-1b, NERF-2, and ELF-1 to the *lyn* promoter Ets site. (a) In vitro translation products of unprogrammed reticulocyte lysate (−) and full-length NERF-1a, NERF-1b, NERF-2, and ELF-1 separated by SDS-PAGE and visualized by autoradiography. (b) DNA binding of full-length NERF-1a, NERF-1b, NERF-2, and ELF-1 in an EMSA using synthetic oligonucleotides coding for the *lyn* promoter Ets site. The full-length NERF and ELF-1 constructs described in Materials and Methods were transcribed in vitro and translated in vitro in a reticulocyte lysate extract. Equal amounts of the translation products as assayed by SDS-PAGE were incubated with the labeled *lyn* oligonucleotide. Lanes 1, 6, and 11 contain the unprogrammed reticulocyte lysate, and lanes 2 to 5, 7 to 10, and 12 to 15 contain the reticulocyte lysates programmed with the NERF and ELF-1 RNAs as indicated above the gel. EMSAs of in vitro-translated NERF-1a, NERF-1b, NERF-2, ELF-1, and unprogrammed reticulocyte lysate incubated with the labeled *lyn* promoter Ets site oligonucleotide probe were carried out with either no competitor (lanes 1 to 5), 50 ng of unlabeled mutant (MUT) *lyn* oligonucleotide (lanes 6 to 10), or 50 ng of wild-type (WT) *lyn* oligonucleotide (lanes 11 to 15). Arrows indicate different DNA-protein complexes.

ELF-1 were in vitro transcribed and translated into protein in a reticulocyte lysate. SDS-polyacrylamide gel electrophoresis (PAGE) analysis of the [^{35}S]methionine-labeled in vitro translation reaction mixtures revealed as the major products proteins with molecular weights slightly higher than those expected (Fig. 6a). This anomalous mobility has been observed for ELF-1, which is a 68-kDa protein and migrates like a protein of more than 90 kDa (Fig. 6a) (81, 87). Because of the similarity of NERF to ELF-1, it is not surprising that NERF migrates at a molecular weight higher than that predicted. Smaller amounts of additional faster-migrating proteins were visible as well in some of the reaction mixtures because of either partial proteolysis, premature translational termination, or alternative internal initiation codons.

To evaluate whether NERF can specifically interact with canonical *ets*-related binding sites, we tested the ability of in vitro-translated full-length NERF proteins to bind specifically to an oligonucleotide containing the human *lyn* promoter Ets binding site (83), which we have recently demonstrated to bind the closely related ELF-1 with high affinity (4). The *lyn* gene encodes a tyrosine kinase which is primarily expressed in B cells and monocytes and is critical for signaling via the B-cell antigen receptor. We compared the abilities of reticulocyte lysates containing the different alternative-splice NERF proteins and of unprimed reticulocyte lysate to form complexes with the *lyn* DNA motif in EMSAs. To ensure equivalent amounts of in vitro-translated protein in each EMSA reaction, we measured the amount of [^{35}S]methionine incorporation and adjusted for the number of methionines present in the different NERF proteins. Molar equivalents of each NERF protein were used for EMSAs. The *lyn* oligonucleotide formed one major complex with proteins present in the NERF-1a, NERF-1b, and NERF-2 extracts (Fig. 6b) which was not formed by the control extract (Fig. 6b). The intensities of the specific protein-DNA complexes were similarly strong for NERF-1a and NERF-1b, but that for NERF-2 was weaker, suggesting that the alternative exons have a slight effect on DNA binding, with NERF-1a and NERF-1b binding more strongly than NERF-2 to the *lyn* promoter probe. Although NERF-2 has a molecular weight higher than those of NERF-1a and NERF-1b, it forms a faster-migrating protein-DNA complex than they do. An additional complex was formed by both the control and all the other extracts. A *lyn* oligonucleotide with a mutation in the core of the Ets binding site (see Materials and Methods) did not compete with the NERF-specific complexes in EMSAs but did compete with the reticulocyte lysate background complex, whereas the wild-type *lyn* oligonucleotide competed with both complexes (Fig. 6b), confirming the sequence specificity of NERF interaction with the *lyn* DNA motif. To compare binding of NERF with binding of ELF-1, we performed an EMSA with in vitro-translated ELF-1 as well. Full-length ELF-1 is able to bind to the lyn site and migrates slightly faster than NERF-1a or NERF-1b, in a manner similar to that of NERF-2, although the molecular weight of ELF-1 is higher than those of NERF-1a and NERF-1b (Fig. 6b). The affinity of ELF-1 towards the *lyn* site is very similar to that of NERF-2, corresponding with the almost identical DNA binding domains in both factors as well as the similarity at the amino terminus and thus suggesting also that the amino terminus may reduce DNA binding affinity.

To analyze the DNA sequence requirements for the binding of NERF and the relative binding affinity, we designed oligonucleotides encoding a whole spectrum of different functionally relevant binding sites for *ets*-related factors, including sites of several B- and T-lymphoid-specific genes (see Materials and Methods) (Fig. 7a). The relative binding affinities and specificities of NERF-1a for these sites were compared with its affinity for the murine *blk* promoter Ets site (4, 19), another high-affinity ELF-1 binding site, in competition experiments. The *blk* gene encodes a tyrosine kinase which is exclusively expressed in B cells and appears to have functions similar to those of *lyn* in antigen receptor signaling. Equivalent amounts of wild-type oligonucleotides were used as competitors in EMSAs with equal amounts of full-length NERF-1a in vitro-translated protein and the *blk* oligonucleotide as a probe (Fig. 7b). The wild-type *blk* oligonucleotide competed effectively with complexes formed by NERF-1a, whereas the mutant *blk* oligonucleotide was unable to inhibit binding of NERF-1a even at high concentrations (Fig. 7b). A 50-ng portion of wild-type competitor completely abolished binding of NERF-1a to

a

ENHANCER	BINDING SITE	NERF-1a
blk	TCCAGGAAGTAT	++++
lyn	ACCAGGAAGTAG	++++
E74	AACCGGAAGTAA	++++
HSV ICP4	GAACGGAAGCGG	++
IgH Enh. π site	GGCAGGAAGCAG	++
IgH Enh. μB site	TTGGGGAAGGGA	++
TdT	GGCAGGAAGCTG	++
mb-1	AACAGGAAGTGA	++
junB	GTCAGGAAGCGC	++
UROKINASE	AGGAGGAAATGA	++
B29	GGCAGGAAGGGG	++
HIV-2 LTR CD3R	GACAGGAACAGC	++
Igκ 3' enh.	TTGAGGAACTGA	+
LCK Prom.	GGCAGGAAGCTT	+
c-FOS SRE	CACAGGATGTCC	+
IL-10 Pu.1	ATGCAGAAGTTC	+
IL-2 NF-AT	AGGAGGAAAAAC	-
HTLV-1 LTR	GGGAGGAAATGG	-
STROMELYSIN Prom.	AGCAGGAAGCAT	-
MSV LTR	GAGCGGAAGCGC	-
CD40	CCCAGGAAGAGG	-
MHC class II Prom.	GTGAGGAACCAA	-
Polyoma PEA3	AGCAGGAAGTGA	-
IL-2 IL-2B	AAGAGGAAAAAT	-
TCR α Enh. Tα2	CAGAGGATGTGG	-
vpreB Prom.	GGGAGGAAGCAC	-
CONSENSUS	TCCAGGAAGTAN AA C	

b

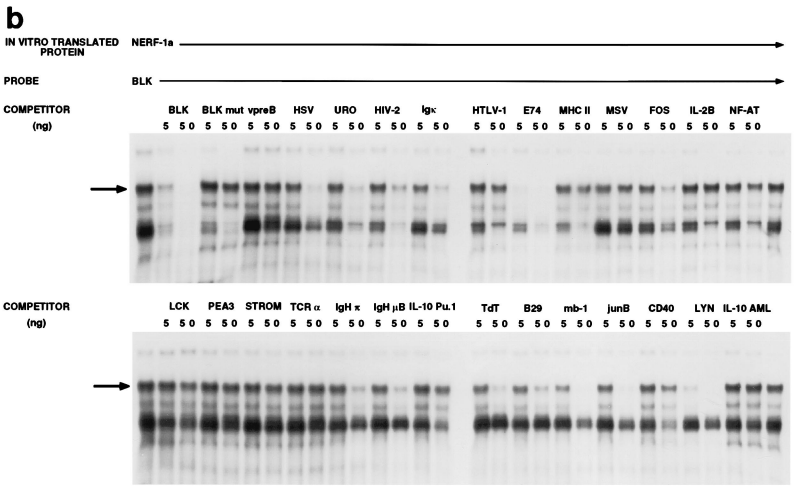


FIG. 7. Interaction of NERF with functionally relevant regulatory sites in different genes. (a) Comparison of the relative binding affinities of NERF-1a for Ets binding sites in the transcriptional regulatory regions of different genes. Sequences present in the regulatory regions of various genes that have been shown to bind Ets-related factors or that correspond to the consensus Ets binding site, i.e., *blk* (19), *lyn* (83), E74 (84), herpes simplex virus (HSV) ICP4 (82), *lck* promoter (51), polyomavirus PEA3 (90), IgH enhancer (Enh.) π and μB sites (53, 54), TdT (21), *mb-1* (31), *junB* (15), *B29* (63), IL-10 Pu.1 (39), CD40 (29), urokinase promoter (61), stromelysin promoter (91), *vpreB* promoter (Prom.) (45), HIV-2 LTR CD3R (50), HTLV-1 LTR (24), murine sarcoma virus (MSV) LTR (30), c-*fos* SRE (16), IL-2 NF-AT (81), T-cell receptor α (TCR α) enhancer Tα2 (35), Ig(κ) 3′ enhancer (68), IL-2B (81), and MHC class II promoter (43) sequences, are shown. The relative DNA binding affinity of full-length NERF-1a towards each site as determined by EMSA (see panel b) is shown on the right. A potential consensus high-affinity binding site for NERF-1a based on this analysis is summarized at the bottom. Capital letters denote nucleotides present in high-affinity binding sites, whereas lower-case letters indicate nucleotides in lower-affinity binding sites. (b) Relative DNA binding activities of NERF-1a for different Ets binding sites in an EMSA using synthetic oligonucleotides coding for the *blk* promoter Ets site as probes. An EMSA of NERF-1a incubated with the labeled *blk* promoter Ets site oligonucleotide probe in the presence or absence of unlabeled competitor oligonucleotides was performed. Assays using in vitro-translated NERF-1a were carried out with either no competitor (lane 1) or 5 and 50 ng of wild-type *blk* oligonucleotide (lanes 2 and 3), mutant (mut) *blk* oligonucleotide (lanes 4 and 5), *vpreB* promoter oligonucleotide (lanes 6 and 7), herpes simplex virus (HSV) ICP4 oligonucleotide (lanes 8 and 9), urokinase promoter oligonucleotide (URO) (lanes 10 and 11), HIV-2 LTR CD3R oligonucleotide (lanes 12 and 13), Ig(κ) 3′ enhancer oligonucleotide (lanes 14 and 15), HTLV-1 LTR oligonucleotide (lanes 16 and 17), E74 oligonucleotide (lanes 18 and 19), major histocompatibility complex (MHC) class II promoter oligonucleotide (lanes 20 and 21), murine sarcoma virus (MSV) LTR oligonucleotide (lanes 22 and 23), c-*fos* (FOS) SRE oligonucleotide (lanes 24 and 25), IL-2 promoter IL-2B oligonucleotide (lanes 26 and 27), IL-2 NF-AT oligonucleotide (lanes 28 and 29), *lck* promoter oligonucleotide (lanes 30 and 31), polyomavirus PEA3 oligonucleotide (lanes 32 and 33), stromelysin promoter oligonucleotide (STROM) (lanes 34 and 35), T-cell receptor α (TCR α) enhancer Tα2 oligonucleotide (lanes 36 and 37), IgH enhancer π site oligonucleotide (lanes 38 and 39), IgH enhancer μB site oligonucleotide (lanes 40 and 41), IL-10 Pu.1 site oligonucleotide (lanes 42 and 43), TdT promoter oligonucleotide (lanes 44 and 45), *B29* promoter oligonucleotide (lanes 46 and 47), *mb-1* promoter oligonucleotide (lanes 48 and 49), *junB* promoter oligonucleotide (lanes 50 and 51), CD40 promoter oligonucleotide (lanes 52 and 53), *lyn* promoter oligonucleotide (lanes 54 and 55), or IL-10 promoter AML-1 site oligonucleotide (lanes 56 and 57). The arrows indicate the position of the specific complex.

the *blk* probe. None of the other *ets*-related binding site oligonucleotides was as effective as the *blk* oligonucleotide in competing for NERF-1a. Only the E74 oligonucleotide encoding the binding site for the *Drosophila melanogaster* E74 Ets factor expressed affinity similar to that of the *blk* site for NERF-1a (Fig. 7b). Oligonucleotides encoding Ets sites in the *lyn* promoter, the *mb-1* promoter, the *junB* promoter, the herpes simplex virus ICP4 promoter, and the urokinase promoter still competed efficiently with NERF-1a. Other sites, including the HIV-2 long terminal repeat (LTR) CD3R site, the IgH enhancer π and μB sites, the *B29* promoter *ets* site, the c-*fos* promoter serum response element (SRE) site, the TdT promoter Ets site, the *lck* promoter Ets site, and the Ig(κ) 3′ enhancer Ets site (Fig. 7b), competed more weakly, leading to partial competition at 50 ng of oligonucleotide. Virtually no competition was observed with the vpreB promoter Ets site, the major histocompatibility complex class II promoter Ets site, the IL-2 promoter IL-2B and NF-AT sites, the HTLV-1 LTR site, the murine sarcoma virus LTR site, the CD40 promoter Ets site, the polyomavirus PEA3 site, and the T-cell receptor α enhancer Tα2 site (Fig. 7b). Figure 6a summarizes the results obtained in this competition analysis, indicating the relative binding affinities of the different sites for NERF-1a and the DNA sequence of the binding core. On the basis of this experiment, we have compiled a putative high-affinity consensus binding site for NERF-1a (Fig. 7a, at the bottom) which is very similar to the consensus recognition sequences for ELF-1 and E74 (87, 89) but divergent from the PU.1 and Ets-1 consensus sequences (68, 87, 89). We compared the relative DNA binding affinity of NERF-1a with the DNA binding affinity of ELF-1 to the different sites (4). NERF-1a and ELF-1 appear to have similar relative affinities for the different Ets sites, suggesting that the DNA binding domain is the primary regulator of DNA binding specificity. These results demonstrate that NERF binds specifically to *ets*-related binding sites recognized by the closely related ELF-1 and that all splice forms of NERF interact with comparable affinity with these sites.

NERF-2, but not NERF-1a and NERF-1b, acts as a transactivator. Since both NERF-1a, NERF-1b, NERF-2, and ELF-1 bind to the same DNA binding sites, we were interested in knowing whether the different splice products of NERF would express any differences in their functions as transcrip-

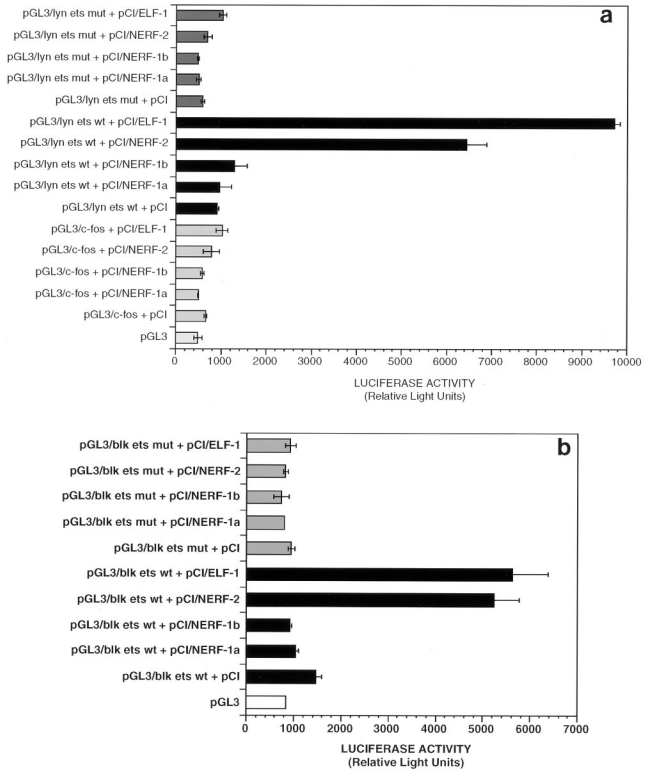


FIG. 8. Transcriptional activation of the *lyn* and *blk* promoter Ets sites by the NERF isoforms and ELF-1. HeLa cells were cotransfected with the indicated NERF or ELF-1 expression vector constructs and luciferase constructs containing two copies of wild-type or mutant *lyn* (a) or *blk* (b) promoter Ets sites as well as the parental pGL3/c-fos promoter construct. Luciferase activity in the lysates was determined 16 h later as described in Materials and Methods. Data shown are means of triplicate measurements from one representative transfection. The experiment was repeated three times with different plasmid preparations with comparable results.

tional regulators and whether NERF and ELF-1 differ in their transactivation capacities. Full-length NERF-1a, NERF-1b, NERF-2, and ELF-1 were inserted into the eukaryotic expression vector pCI and cotransfected into HeLa cells together with a pGL3 reporter gene construct containing the luciferase gene in which two copies of the *lyn* promoter Ets binding site or of the *blk* promoter Ets binding site were inserted upstream of the minimal c-*fos* promoter (23). Δ56-pGL3 containing only the minimal c-*fos* promoter expressed very little luciferase activity above the background of the parental promoterless pGL3 vector (Fig. 8). Two copies of the wild-type *lyn* promoter Ets site were only slightly stronger than the minimal c-*fos* promoter, whereas a construct containing the same copy number of the mutant *lyn* promoter Ets site was transcriptionally inactive. Cotransfection with NERF-1a, NERF-1b, NERF-2, and ELF-1 expression vectors resulted in an 8-fold transcriptional stimulation of the wild-type *lyn* promoter Ets site by NERF-2 and a 12-fold stimulation by ELF-1, whereas NERF-1a and NERF-1b were virtually inactive in transactivation (Fig. 8). Mutation of the *lyn* promoter Ets site practically abolished transactivation by NERF-2 or ELF-1, confirming the specificity of transactivation. Two copies of the wild-type *blk* promoter Ets site were almost inactive in the absence of NERF or ELF-1. Cotransfection with NERF-1a, NERF-1b, NERF-2, and ELF-1 expression vectors resulted in a fourfold transcriptional stimulation of the wild-type *blk* promoter Ets site by NERF-2 and a fivefold stimulation by ELF-1, whereas NERF-1a and NERF-1b were inactive in transactivation (Fig. 8). Mutation of the *blk* promoter Ets site abolished transactivation by NERF-2 or ELF-1. Similar results were obtained with COS and CV-1 cells (data not shown). Thus, two B-cell tyrosine kinase genes contain high-affinity binding sites for NERF and ELF-1 which can be transactivated by these two Ets factors.

To confirm that the lack of transactivation capability of NERF-1a and NERF-1b is not due to the lack of expression or reduced levels of expression in transfected cells, we performed EMSAs using aliquots of the cell extracts from transfected cells used in luciferase assays (Fig. 9). Extracts from cells transfected with the parental pCI expression vector formed only weak protein-DNA complexes. NERF-1a, NERF-1b, and NERF-2 formed complexes with mobility similar to that of the in vitro-translated NERF isoforms. Again NERF-1a and NERF-1b migrated with mobility slower than that of NERF-2. The amounts of NERF-1a and NERF-1b were similar to or slightly higher than those of NERF-2, suggesting that the inability of NERF-1a and NERF-1b to transactivate is not due to a lack of expression or to reduced expression. ELF-1 formed a very strong complex with mobility similar to that of NERF-2 and appeared to be more highly expressed than the different isoforms of NERF. Additional faster-mobility complexes were observed for both NERF and ELF-1 extracts, potentially indicating partial proteolysis. Again NERF-1a and NERF-1b contained larger amounts of these products than did NERF-2. Interestingly, the apparent NERF-2 proteolysis product migrated with mobility slower than that of NERF-1a or NERF-1b and NERF-1b was slower than NERF-1a, indicating that proteolysis most likely clipped part of the carboxy terminus. This suggests that the carboxy terminus contributes to the anomalous mobility of full-length NERF-2 compared with NERF-1a and NERF-1b. We conclude from these experiments that NERF-2 can stimulate transcription in a sequence-specific

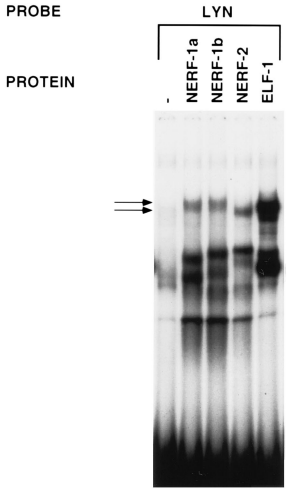


FIG. 9. DNA binding analysis with whole-cell extracts from COS cells transfected with NERF-1a, NERF-1b, NERF-2, and ELF-1 expression vectors in an EMSA using synthetic oligonucleotides coding for the *lyn* promoter Ets site. NERF and ELF-1 pCI expression vector DNA (1.5 μg) was transfected into COS cells as described in Materials and Methods. Equal amounts of whole-cell extract were incubated with the labeled *lyn* oligonucleotide. Lane 1 contains extract from cells transfected with the parental pCI vector, and lanes 2 to 5 contain the extracts from cells transfected with the NERF and ELF-1 expression vectors as indicated above the gel. Arrows indicate DNA-protein complexes formed by the full-length NERF and ELF-1 proteins.

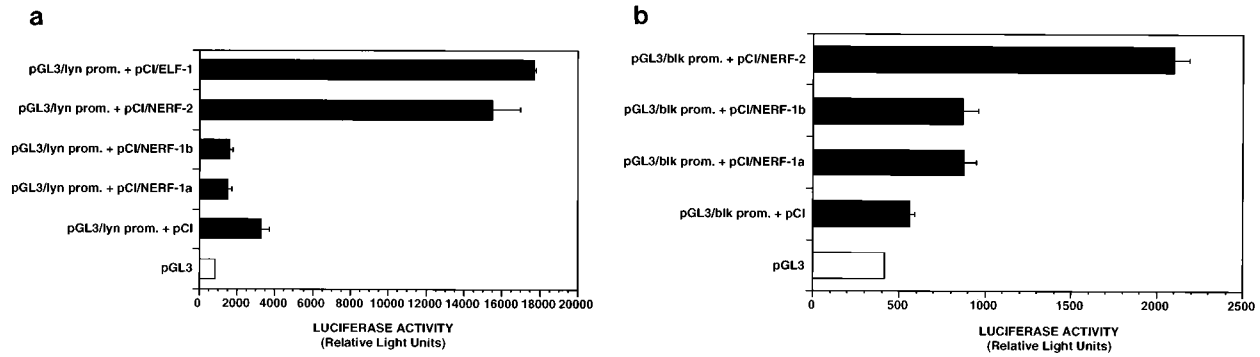


FIG. 10. Transcriptional activation of the *lyn* and *blk* promoters by the NERF isoforms and ELF-1. HeLa (*lyn*) or COS (*blk*) cells were cotransfected with the indicated NERF or ELF-1 expression vector constructs (1.5 μg) and luciferase constructs containing the *lyn* (a) or *blk* (b) promoter (3.5 μg) as well as the parental pGL3 vector. Luciferase activity in the lysates was determined 16 h later as described in Materials and Methods. Data shown are means of triplicate measurements from one representative transfection. The experiment was repeated four times with different plasmid preparations with comparable results.

manner and that the amino terminus of NERF-2 contains at least part of the transactivation domain, since the alternatively spliced forms NERF-1a and NERF-1b lack most of the transactivation ability, even though they are expressed at levels similar to those of NERF-2. The results, furthermore, suggest that NERF-2 and ELF-1 act as positive transcriptional regulators and express similar transactivation capacities. Although NERF-1a and NERF-1b bind with affinities comparable to or slightly higher than those of NERF-2 and ELF-1 to the *lyn* promoter Ets site, they differ from NERF-2 and ELF-1 in their capacity to transactivate the *lyn* and *blk* promoter Ets sites.

The *lyn* and *blk* genes are potential B-cell targets for NERF-2. Sequence analysis of promoter regions of the human *lyn* and murine *blk* genes revealed the presence of DNA motifs similar to *ets*-related binding sites, and we demonstrated that NERF-2 can transactivate the isolated *lyn* and *blk* promoter *ets* sites. To determine whether the *lyn* and *blk* promoter *ets* sites are targets for NERF in the context of the *lyn* and *blk* promoters, we cloned the promoter regions of the human *lyn* gene and of the murine *blk* gene upstream of the luciferase gene into the pGL3 luciferase vector (4). The *lyn* promoter luciferase construct was cotransfected with the NERF-1a, NERF-1b, NERF-2, and ELF-1 expression vectors into HeLa cells (Fig. 10a). The *lyn* promoter expressed an approximately threefold increase in luciferase activity over the basal pGL3 vector in HeLa cells. Both NERF-2 and ELF-1 induced *lyn* promoter transcription six- to sevenfold. However, NERF-1a and NERF-1b were unable to induce transcription of the *lyn* promoter and actually decreased the basal level of the *lyn* promoter by 50% to almost the background level, suggesting that NERF-1a and NERF-1b may potentially act as inhibitors of the *lyn* promoter. Similarly, cotransfection of the *blk* promoter luciferase construct with the different NERF expression vectors into COS cells led to a fourfold transactivation by NERF-2 and a marginal enhancement by NERF-1a and NERF-1b (Fig. 10b). The *blk* promoter by itself was only weakly active in COS cells because of a lack of crucial B-cell-specific transcription factors such as B-cell-specific activator protein (BSAP). These results support the notion that the *lyn* and *blk* genes might be targets for NERF in B cells and that NERF and ELF-1 might be involved in the regulation of the same or similar genes.

DISCUSSION

In our search for transcriptional regulators of the immune system we have isolated a gene for a novel member of the Ets transcription factor/oncogene family, NERF, with at least four alternative splice products, i.e., NERF-1a, NERF-1b, NERF-2 (now renamed NERF-2a), and NERF-2b, which differ in their amino termini. Comparison of the deduced amino acid sequence of NERF with those of other members of the Ets family reveals that the level of homology to ELF-1, which has been demonstrated to regulate various T- and B-cell-specific genes, is highest. Homologies are clustered in the putative DNA binding domain in the middle of the protein, a basic domain just upstream of this domain, and several shorter stretches of homology towards the amino terminus. NERF-2 contains additional homology to ELF-1 not present in NERF-1 at its amino terminus. Northern blot analysis indicates the presence of two predominant NERF transcripts in various tissues, with the highest levels in testis, ovary, skeletal muscle, spleen, thymus, heart, kidney, lung, and liver. Although both NERF-1a and NERF-1b bind to DNA with affinity higher than that of NERF-2, only NERF-2 acts as a potent transactivator.

Our results demonstrate that alternative splicing of NERF transcripts generates at least four mRNAs which are coexpressed in B and T cells, spleen, and fetal liver. We do not know whether the same splice products are expressed in other cell types and tissues as well; however, renal epithelial cells express only two of these transcripts, and on the basis of Northern blot analysis the two major transcripts appear to be differentially expressed in different tissues. The alternative splice products encode proteins which differ in their amino termini. Since the DNA binding domain is unchanged in all NERF splice products, it is not surprising that the isoforms bind with similar specificities to DNA. The NERF isoforms exhibit, however, slightly different affinities towards DNA and strikingly distinct transcriptional activation potentials. The amino-terminal 158 amino acids of NERF-2 are highly acidic (>22%), and amino acid sequence comparison demonstrates homology to an acidic transactivation domain in ELF-1 located in the amino-terminal 150 amino acids (88). In particular, domains A, B, and C are conserved in NERF and ELF-1, indicating an apparent important function in both proteins. Part of this acidic domain including the conserved domain A and part of domain B is absent in NERF-1a and NERF-1b and replaced by an alternative exon encoding 19 amino acids unrelated to ELF-1 or NERF-2. NERF-1a and NERF-1b lack significant transactivation capabilities under the experimental conditions used in this study, even though they are expressed at levels similar to those of NERF-2 in transfected cells, whereas NERF-2 acts as

a transcriptional activator with strength similar to that of ELF-1, indicating a role for the amino terminus of NERF-2 in transactivation. The apparent distinct transactivation capacities of the NERF isoforms suggest distinct biological functions. NERF and ELF-1 are coexpressed in various tissues and cell types, including B and T cells, and the DNA binding domains of NERF and ELF-1 are remarkably similar, indicating that both factors might be involved in the regulation of the same genes. Indeed, NERF and ELF-1 bind to the same Ets sites in a variety of B- and T-cell-specific genes with comparable affinities and one of the isoforms of NERF, NERF-2, transactivates the *lyn* and *blk* promoter Ets sites as well as ELF-1 does, whereas the two other NERF isoforms, NERF-1a and NERF-1b, appear inactive as transactivators. Interestingly, although NERF-1a and NERF-1b lack transactivation capacities, they apparently bind more strongly to DNA than do equal amounts of NERF-2 or ELF-1. RNase protection assays suggest that NERF-1a, NERF-1b, NERF-2a, and NERF-2b are expressed at similar levels in several murine pre-B-cell and mature B-cell lines (Fig. 5) (62). These results lead to several hypotheses for NERF and ELF-1 function, including the following. (i) In vivo only one of these factors binds to the promoter or enhancer sites because of the presence of other regulatory elements surrounding the Ets site and protein-protein interactions as well as differential phosphorylation leading to selection of either one of the isoforms of NERF or ELF-1. (ii) Redundancy exists so that both factors can exert the same effect, although under slightly different circumstances. (iii) NERF and ELF-1 are both involved in regulation of these Ets sites, but NERF-1a and NERF-1b exert effects opposing those of NERF-2 and ELF-1. NERF-2 and ELF-1 act as positive regulators, and NERF-1a and NERF-1b act as negative regulators. Differential phosphorylation or protein-protein interactions during cell differentiation or activation could shift the equilibrium from positive to negative regulation or vice versa. A paradigm for the last hypothesis involving two antagonistic Ets factors, pointed and yan, acting via the same regulatory element exists in *Drosophila* eye development (47, 64, 75). During differentiation of R7 retinal cells MAP kinase activation leads to phosphorylation of two members of the Ets family, pointed and yan. yan in the unphosphorylated form inhibits transcription induced by pointed and cannot activate transcription. Upon phosphorylation by MAP kinase yan becomes derepressed because of a change in stability and subcellular localization and pointed becomes activated, thus leading to transcriptional activation of the specific target genes (64, 75). A similar scenario involving differential phosphorylation and/or differential protein-protein interactions which would shift the equilibrium between positive and negative regulation could be envisioned for the function of the different NERF isoforms and ELF-1. Selection of a particular isoform of NERF or ELF-1 for a specific Ets binding site involves most likely a combination of availability of each factor due to expression in the cell, affinity to a specific DNA motif, phosphorylation, and protein-protein interactions dependent on regulatory elements surrounding the Ets site in a particular gene leading to distinction between ELF-1 and different isoforms of NERF in a specific environment. In some of our transactivation studies, NERF-1a and NERF-1b indeed appeared to act as inhibitors, reducing the basal transcription level by ~50%.

Transactivation capability of ELF-1 is at least partially regulated by the interaction of ELF-1 with hypophosphorylated retinoblastoma protein Rb (88, 94). Rb inhibits transactivation induced by ELF-1. Upon phosphorylation of Rb during the cell cycle ELF-1 is released and derepressed. The Rb protein binds close to the amino terminus of ELF-1, presumably to a domain involving the Rb binding consensus sequence LXCXE (88). NERF-2 contains a region of similarity to this domain; however, the LXCXE motif is not conserved in NERF-2, although there are several stretches of striking sequence homology (domains A and B) between NERF-2 and ELF-1 in this region. This suggests that either NERF-2 does not bind to Rb or that the LXCXE motif is not crucial for Rb binding. Computer-assisted sequence comparison of domains conserved in both NERF-2 and ELF-1 with the GenBank database does not reveal any homology to any other known proteins, leaving the question open as to the potential function of these conserved domains. Since a transactivation domain is most likely located at the amino terminus of NERF-2, the conserved domains might be involved in either transactivation, posttranslational modifications, or protein-protein interactions. The relevance of these homologies for the biological function of NERF or ELF-1 has to be determined.

NERF-1a and NERF-1b are isoforms which differ by an insertion of 12 amino acids within the amino terminus. Transcripts with and without this insertion have been observed in various tissues and cell lines. The insertion does not appear to affect DNA binding or transactivation, but it contains a potential MAP kinase or jun kinase phosphorylation site suggesting that it may serve as a phosphorylation site regulating NERF activity upon activation of the MAP kinase or jun kinase pathway by specific stimuli. Since neither NERF-1a nor NERF-1b appears to transactivate under the conditions used in this study, phosphorylation might be one possible mechanism of modulating their transactivation capacities. NERF-1a and NERF-1b appear to act as potential inhibitors of ELF-1, and NERF transcriptional activity and phosphorylation may influence DNA binding, protein stability, and/or cellular localization as seen for another repressor Ets factor, yan (75). Both NERF and ELF-1 contain multiple putative MAP kinase or jun kinase phosphorylation sites, most of them located in the amino-terminal 200 amino acids. Several of these putative phosphorylation sites are conserved between NERF and ELF-1, indicating a potential regulatory function.

We have detected high-affinity binding sites for NERF in the regulatory regions of various B- and T-cell-specific genes, especially in the promoter regions of the specialized hematopoietic tyrosine kinase genes *lyn* and *blk*, and we have demonstrated that one of the NERF isoforms, NERF-2, can specifically transactivate the *lyn* and *blk* promoters as well as the isolated *lyn* and *blk* promoter Ets sites. The same sites also bind ELF-1 with high affinity, and ELF-1 is able to transactivate these sites, suggesting that the *lyn* and *blk* genes represent B-cell targets for NERF and ELF-1. blk and lyn are important B-cell signal-transducing components whose expression is tightly controlled and tissue restricted. *blk* is expressed in pre-B cells and mature B cells but not in plasma cells, in a manner similar to that of expression of *mb-1* and *B29*, which also contain binding sites for NERF and ELF-1 in their respective promoters (19). Very little is known about regulation of *blk* gene expression except that the B-cell-specific transcription factor BSAP plays an important role (19). *lyn* is primarily expressed in myeloid and B cells. The *lyn* promoter has not been analyzed in detail. Our results present the first evidence that the Ets factors NERF and ELF-1 might be involved in B-cell regulation of *lyn* and *blk* gene expression. Although both NERF and ELF-1 are expressed in a variety of tissues, ELF-1, for example, has been shown to be an important transcription factor for T-cell-specific gene regulation (49, 50, 86, 87). Thus, the involvement of NERF as well as ELF-1 in B-cell gene regulation may be a consequence of several mech-

anisms, including among others differential expression and function of the different isoforms, B-cell-specific phosphorylation events, and interactions with B-cell-specific transcription factors.

In conclusion, we have established NERF as a new member of the Ets family with significant sequence and functional homology to ELF-1 and we have provided evidence that NERF can act as a transactivator of B-cell genes. The expression of several isoforms of NERF with strikingly distinct transactivation capacities adds an additional level of complexity as to the biological role of NERF. Since NERF is expressed in several different tissues and cell types, it is likely that it plays an important role in regulation of a whole set of genes outside the immune system as well. Further analysis of function and structure-function relationships of NERF will provide insights into the mechanisms of action of NERF. Since Ets factors have been implicated in the regulation of many tissue- and development-specific genes as well as in human tumorigenesis, further studies as to the involvement of NERF in cell differentiation, gene regulation, and tumorigenesis are warranted.

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