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elk, Tissue-Specific *ets*-Related Genes on Chromosomes X and 14 near Translocation Breakpoints

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The *myb-ets*–containing acute leukemia virus, E26, transforms myeloblasts and erythroblasts in culture and causes a mixed erythroid and myeloid leukemia in chicks. Genes (*ets*-1, *ets*-2, and *erg*) with variable relatedness to the v-*ets* oncogene of the E26 virus have been identified, cloned, and characterized in several species. Two new members (*elk*-1 and *elk*-2) of the *ets* oncogene superfamily have now been identified. Nucleotide sequence analysis of the *elk*-1 cDNA clone revealed that this gene encodes a 428-residue protein whose predicted amino acid sequence showed 82% similarity to the 3′ region of v-*ets*. The *elk* or related sequences appear to be transcriptionally active in testis and lung. The *elk* cDNA probe detects two loci in the human genome, *elk*-1 and *elk*-2, which map to chromosome regions Xp11.2 and 14q32.3, respectively. These loci are near the translocation breakpoint seen in the t(X;18) (p11.2;q11.2), which is characteristic of synovial sarcoma, and the chromosome 14q32 breakpoints seen in ataxia telangiectasia and other T cell malignancies. This suggests the possibility that rearrangements of *elk* loci may be involved in pathogenesis of certain tumors.

E26 IS A REPLICATION-DEFECTIVE retrovirus whose genome harbors two oncogenes, v-*myb* and v-*ets* (*1, 2*). E26 causes erythroblastosis and myeloblastosis in chickens and transforms myeloblasts, erythroblasts, and quail embryo fibroblasts in culture (*3–5*). Beug *et al.* (*6*) have shown that the v-*myb* domain of E26 causes myeloblast transformation and that v-*ets* is responsible for erythroblast and fibroblast transformation. The v-*myb* and v-*ets* oncogenes are expressed as a 135-kD polyprotein (p135 *gag-myb-ets*) that is localized in the nucleus of transformed cells (*7–9*) and exhibits DNA binding activity (*10*). Sequences homologous to v-*ets* are present in the genome of organisms widely separated phylogenetically, namely, chicken [Ck-c *ets*-1 and -2 (*11–13*)], mouse [M-*ets*-2 (*14*)], human [Hu-c *ets*-1 and -2, as well as *erg* (*14–18*)], sea urchin [Su(LV)-*ets*-2 (*19*)] and *Drosophila* [D-*ets*-2 (*20*)]. In humans, *ets*-1 and *ets*-2 and *erg* have been mapped near chromosome regions (*1, 18, 21*) that are involved in translocations characteristic of certain leukemias and lymphomas (*22–25*). In this study, we report the molecular cloning, chromosomal localization, and expression of two *ets*-like genes *elk*-1 and *elk*-2.

A COLO 320 cDNA library in the λ gt10 vector (*17, 18*) was screened with Hu-*ets*-2 cDNA (*26*). A clone designated λ 11 was found that contained *elk*-1 sequences (Fig. 1A). *elk*-1 cDNA, which is derived from the *elk*-1 locus, detects a closely related unlinked gene, *elk*-2. The *elk*-1 cDNA clone is ~2.9 kb in length. A computer analysis of the nucleotide sequence revealed that the 5′ region of *elk*-1 (nucleotides 306 to 603) showed ~67% homology with only the 3′ region (nucleotides 2044 to 2334) of the v-*ets* oncogene. Thus, the *elk*-1 cDNA identified a single domain of similarity with the v-*ets* oncogene: this is unlike Hu-*ets*-2 and *erg*, which have two v-*ets*–related domains. The deduced amino acid sequence of *elk*-1 cDNA is ~82% homologous in its 5′ region with the 3′ end of v-*ets* (Fig. 1B). The longest open reading frame in *elk*-1, starting with a methionine codon at position 316 in the nucleotide sequence (Fig. 1A), could encode a 428–amino acid polypeptide with an estimated relative molecular mass of ~45 kD. Since a stop codon is present in the same frame farther upstream (Fig. 1A), the ATG at position 316 may be used as the initiation codon. The initiation codon at residue 316 is preceded by another initiation codon (nucleotide 115) in the same reading frame upstream of the termination codon (nucleotide 277) that precedes the long open reading frame. Thus, a potential polypeptide composed of 54 amino acids could also be synthesized from *elk*-1 mRNA in the bicistronic manner proposed for some eukaryotic mRNAs. A similar phenomenon was observed in *erg*-1 and *erg*-2 mRNAs (*17, 18*). There are four in-frame ATGs located in the 5′ portion of the sequence, one of which essentially conforms to the Kozak consensus sequence (*27*).

The deduced amino acid sequence of the *elk*-1 polypeptide shows that it has a primary length of 428 amino acids and is rich in proline (62 residues), serine (44 residues), leucine (42 residues), glycine (40 residues), and alanine (37 residues). It bears one potential glycosylation site at amino acid position 51. A plot of hydropathicity values, as determined by the method of Kyte and

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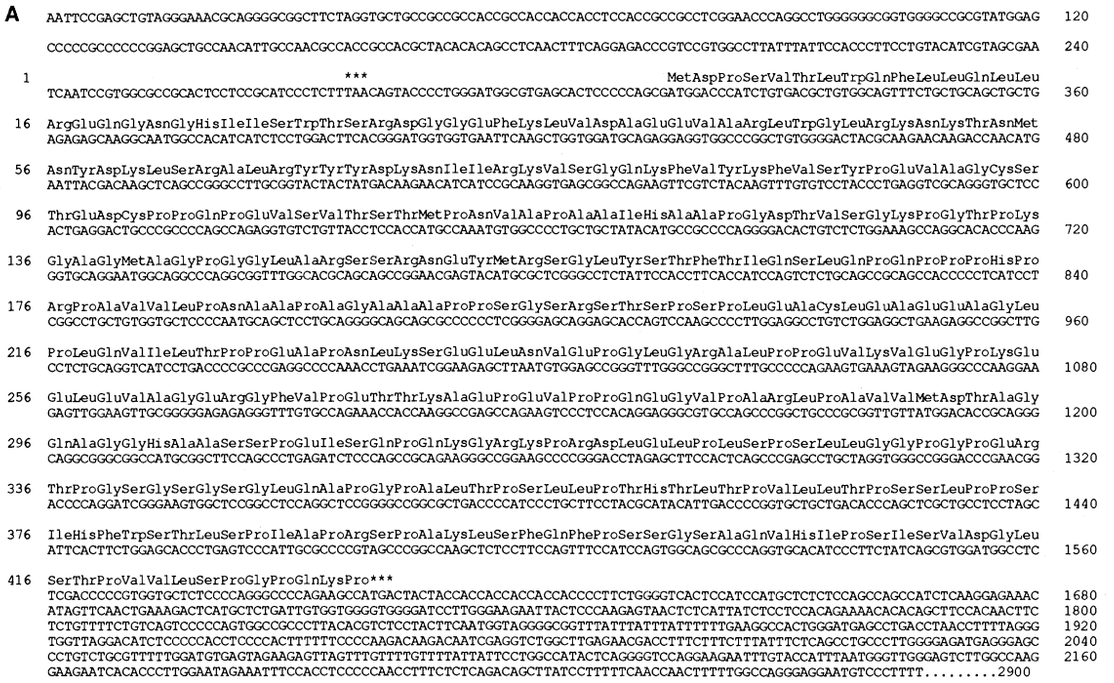

Fig. 1. (**A**) The nucleotide sequence and deduced amino acid sequence of *elk*-1 cDNA clone (λ 11). Sequence analysis was carried out by the dideoxy chain termination method (*43*). The nucleotide positions are indicated on the right and amino acid positions are indicated on the left. The termination codons in-frame with the reading frame upstream and downstream are indicated by asterisks. (**B**) Comparison of the predicted amino acid sequence of *elk*-1 with v-*ets* (*1, 2*), c-*ets*-1a (*15*), c-*ets*-2 (*16*), and *erg*-1 (*17*). Solid region represents identical amino acids. Dots were used for maximal alignment of the amino acid sequences. Single-letter abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

Doolittle (*28*), reveals that the amino- and carboxyl-terminal regions are hydrophobic, and the remainder of the protein is hydrophilic.

Comparison of the amino acid sequences specified by *elk*-1 to those specified by v-*ets*, Hu-c-*ets*-1, Hu-*ets*-2, and *erg* revealed ~76% homology over a range of 83 amino acids (positions 5 to 86) (Fig. 1B); if one takes into consideration conservative amino acid replacement, the homology in this region is 81 to 82%. Thus, unlike Hu-*ets*-2 (*16*) and *erg* (*17*), the *elk*-1 gene has only a single domain of homology with the v-*ets* oncogene. The domain of homology between *elk*-1 and v-*ets* is conserved among all members of the *ets* gene family, suggesting that this domain performs a common biochemical function or functions (Fig. 1B). Our results suggest that *elk*-1, *erg*-1, *erg*-2, *ets*-2, *ets*-1, and v-*ets* are all DNA binding proteins and that this structurally related (conserved) domain of these proteins is partly responsible for DNA binding activity (*26*).

A computer-assisted search of the NBRF protein database revealed that the amino acid sequences specified by *elk*-1 exhibited homology with several proteins. If one takes conservative amino acid replacement into consideration, the *elk*-1 protein exhibited ~35 to 44% homology (over a stretch of ~426 amino acids) to human collagen α1 chain, human *myc* protein, progesterone receptor, basic proline-rich protein precursor (human), and probable nuclear antigen of Epstein-Barr virus (EBV). Furthermore, the sequence Gly-Pro-X (where X represents any of the other amino acids) is repeated six times in the entire sequence (Fig. 1A), as in the collagen molecule where it is repeated more than 100 times. The sequence Pro-Pro-X is repeated seven times and the sequence Leu-X_{3-5}-Leu is repeated several times in the entire sequence (Fig. 1A). The significance of these amino acid sequence similarities and repeats remains to be established.

To confirm the presence of the deduced open reading frame for *elk*-1 protein in cDNA clone λ 11, the entire insert was subcloned in the GEM riboprobe vector (Promega Biotec), downstream from a recognition site for the RNA polymerase of bacteriophage SP6. To show that *elk*-1 protein presumably initiates from the ATG codon at nucleotide position +316, we deleted the sequences (above nucleotide +413) that code for this methionine initiation codon and cloned in the plasmid vector KS downstream from the T7 RNA polymerase promoter. For further confirmation of the open reading frame of *elk*-1, sense RNA was made from DNA constructs in which the open reading frame was truncated by the use of restriction enzymes Bgl II and Bam HI. The RNAs synthesized in vitro by the T7 and SP6 RNA polymerase were then translated in a rabbit-reticulocyte lysate in the presence of [^{35}S]methionine. The largest protein bands detected migrated in SDS–polyacrylamide gel electrophoresis with an apparent molecular mass of approximately 58 kD (Fig. 2, lane 2). This value is much higher than what is predicted from the nucleotide sequence (45 kD). The high proline content of the *elk*-1 protein may have caused an ~13 kD increase, as a similar phenomena have been observed with other proline-rich pro-

teins such as those encoded by Ckc-ets-1 (*11*), *myc*, *myb*, and *bcl*-2 (*29*). The Bam HI–truncated sense RNA produced proteins of the same size as the full-length clone, confirming that the open reading frame extended within the Bam HI site (Fig. 2, lane 3). The Bgl II cut and the minus ATG sense RNA yielded proteins smaller than 58 kD (Fig. 2, lanes 5 and 4) confirming that the 58-kD proteins are derived from the *elk*-1 open reading frame initiating at residue 316 shown in Fig. 1A. No protein was synthesized from RNA transcribed from a clone with *elk*-1 cDNA in the opposite orientation.

In parallel experiments, the *elk*-1 probe E2.5 was used to determine the chromosome location of the human *elk* locus. DNAs from a panel of rodent human somatic cell hybrids (*30*) that retained overlapping subsets of human chromosomes were analyzed for the presence of *elk* genomic loci by Southern blot hybridization analysis. As shown in Fig. 3A, murine DNA (lane 1) exhibits two *elk*-specific Sst I fragments (5.4 and 4.1 kbp), whereas human DNA (in lane 2) gives three Sst I bands at 17, 15 (not resolved from 17 in lane 2), and 2.8 kbp. Lanes 3 to 18 (Fig. 3A) contain DNAs from mouse-human hybrids; the 2.8-kbp *elk*-specific fragment is present in lanes 5, 9, and 13 to 18, which contain DNA from hybrids retaining the human X chromosome and is absent in hybrids (lanes 3, 4, 7, 8, and 10 to 12), which do not retain the human X chromosome. The 17-kbp human band is retained in hybrids containing chromosome 14 (lanes 4, 5, 9 to 12, 14, and 16 to 18) and is absent in hybrids without chromosome 14 (lanes 3, 6 to 8, 13, and 15). The 15-kbp band segregates with the 2.8-kbp band (see especially lanes 13 and 15) and thus is X-linked. Presence of *elk* loci, hereafter referred to as *elk*-1 and *elk*-2, on X and 14 respectively, was confirmed with the aid of additional enzymes (Hind III and Eco RI) and hybrids. Results shown in Fig. 3, A and B, illustrate a regional localization of the *elk*-2 locus on chromosome 14. Hybrid 2S9 (Fig. 3A, lane 4) is missing the terminal region of chromosome 14 from the JH region of the IgH locus (at 14q32.3) to 14qter and is positive for *elk*-2; whereas the hybrid AB3 (Fig. 3B, lane 5) retains 14q11→14qter and is also positive for *elk*-2. Thus *elk*-2 is between the TCRα locus (at 14q11) and the IgH locus (at 14q32.3). Figure 3B illustrates a narrower regional localization for *elk*-1 and -2. The rat-human hybrid 52-63cl7 (Fig. 3B lane 3) carries a 14q+(14pter→14q32::Xq13→Xqter chromosome) in which the 14q32 break is centromeric to an arbitrary DNA probe, D14S1, located at 14q32.1→14q32.2 (*31*) and was negative for both *elk* genes. Thus, *elk*-1 is not in region Xq13→Xqter as confirmed by absence of *elk*-1 sequences in hybrid MCP6 (Fig. 3B, lane 4), which also retains the region Xq13→Xqter (*32*). In summary, somatic cell hybrid analysis assigns *elk*-1 to Xpter→Xq13 and *elk*-2 to 14q32 centromeric to the IgH locus. In situ hybridization of ^{3}H-labeled *elk*-1 probe (E2.5) to normal human metaphase chromosomes (*30, 33*) confirmed and refined these localizations to chromosomes X and 14. After autoradiography and chromosome banding (*33*) metaphase spreads were analyzed for grain distribution. Over 16% of the grains were at band 14q32 with most grains

Table 1. Sizes of the *elk* transcripts observed in different cell lines.

Cells	Probe	Major bands (kb)	Minor bands (kb)
COLO 320	E2.5	~3.1	~7.2, 2, 1.7
	E0.4	~4.6, 3.1	~2.3, 1.6
MOLT 4	E2.5	~3.1	
	E0.4	~4.6	~1.6 to 3.1
K562	E2.5	~3.1	
	E0.4	~0.9 to 4.7	
EL 4	E2.5	~2.8	
	E0.4	~4.1, 2.9	~6
L-M(TK$^-$)	E0.4	~5.8, 4.1, 2.9, 1.8	
CEF	E2.5	~1.6	
	E0.4	~4.4, 0.9 to 1.8	

The *elk*-1 probes (E2.5 and E0.4) used are described in the legend to Fig. 4.

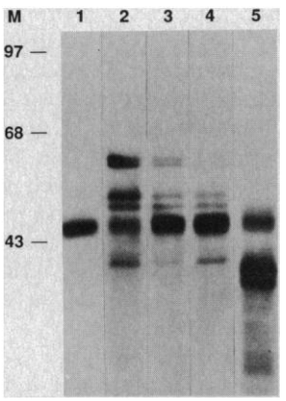

Fig. 2. In vitro synthesis of *elk*-1 proteins. The *elk*-1 cDNA clone [cloned in riboprobe GEM and KS vectors; pSP6 *elk*-1 and pT7 *elk*(2.5 kb)] were linearized by digestion with Hind III, Bam HI or Bgl II and transcribed in vitro with SP6 and T7 polymerase (Promega) according to the manufacturer's protocols. The uncapped RNAs produced were translated in vitro with a rabbit reticulocyte lysate (Promega) in the presence of [^{35}S]methionine. The labeled proteins were subjected to electrophoresis on a 12% SDS–polyacrylamide gel (*44*) and revealed by fluorography. Lane 1, control minus RNA, lanes 2, 3, and 5, translation products of RNAs transcribed from pSP6 *elk*-1 cut with Hind III, pSP6 *elk*-1 cut with Bam HI, and pSP6 *elk*-1 cut with Bgl II. Lane 4, translation products of RNA transcribed from pT7 *elk*-1 (2.5 kb) cut with Hind III. Lane M, molecular weight standards in kilodaltons.

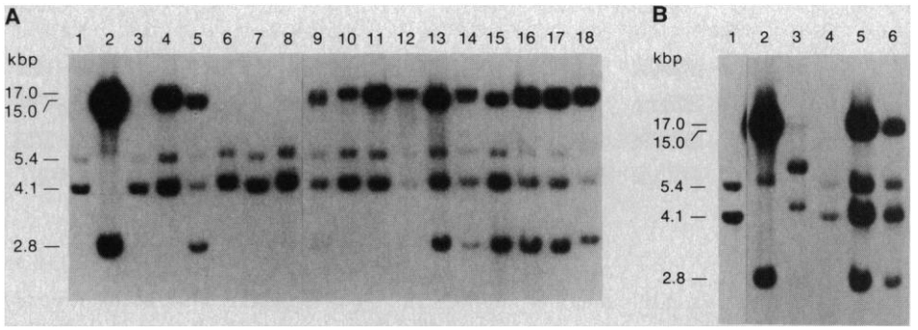

Fig. 3. The *elk*-1 probe E2.5 detects two human genomic loci *elk*-1 and *elk*-2, which map to chromosome region Xpter→Xq13 and 14q32, respectively. Previously characterized hybrid cell DNAs (*30*), were analyzed for presence of *elk* loci. (**A**) DNA (~10 μg per lane) from (lane 1) mouse; (lane 2) human; (lane 3) hybrid cl31 retaining human chromosome 17; (lane 4) hybrid 2S9 retaining partial chromosomes 4, 8, 12, 13, and 14pter→14q32; (lane 5) hybrid 77-31 retaining 1, 3, partial 4, 5 to 9, partial 10, 13, 14, 17, and 20-X; (lane 6) hybrid cl21 retaining chromosome 7; (lane 7) hybrid PB5 retaining partial 1, partial 2, partial 3, 5q, 8, 11q, and 17; (lane 8) hybrid N9 retaining 6, 7, partial 17, and 21; (lane 9) hybrid GL-5 retaining 4, 8, 13, 14, partial 17, 18, 20, 21, partial 22 and X; (lane 10) hybrid 3a-9 retaining partial 4, 6, 12, partial 13, 14, 17, 21, and 22; (lane 11) hybrid 8c retaining 4, partial 6, partial 8, 9, 12, partial 13, 14, 15, 17, 21, and 22; (lane 12) hybrid GL3 retaining partial 3, 4, 6, 7, 12q, 14, 15, and 17 to 20; (lane 13) hybrid AA3 retaining partial 4 and X; (lane 14) hybrid BD3 retaining 1 to 8, 10 to 16, and 15 to X; (lane 15) hybrid G5 retaining partial 4, 6, 12, 20, and X; (lane 16) S5 retaining 3, 5, partial 6, 7, 9, 11, 13, partial 14, 15, 17, 18, partial 22, and X; (lane 17) S3 retaining 3, 5, 6, 9, 14, 15, 17, 22, and X; (lane 18) 77-30 retaining partial 1, 3, partial 4, 5 to 7, 9, partial 10, 13, 14, 17, and 20 to X. (**B**) DNA (~10 μg per lane) from (lane 1) mouse; (lane 2) human; (lane 3) rat-human hybrid 52-63 cl7 retaining a 14q$^+$ (14pter→14q32::Xq13→Xqter) chromosome; (lane 4) hybrid MCP6 retaining a 6p$^+$ (6qter→6p21::Xq13→Xqter) chromosome; (lane 5) hybrid AB3 retaining chromosomes 6, 20q$^+$ (20pter→20q13::14q11→14qter), and X; (lane 6) hybrid AA3 retaining partial 4 and X. DNAs were digested with restriction endonuclease Sst I, electrophoresed, transferred to nylon filter, and hybridized to ^{32}P-labeled *elk*-1 probe E2.5 [~2.5-kb Eco RI fragment of the λ 11 cDNA as previously described (*30*)]. Filters were washed in 0.1× saline sodium citrate and 1% SDS, at 65°C, and exposed to x-ray film.

at 14q32.3 and more than 17% of the Xp grains were between Xp22.1 and Xp11 with most grains at Xp11.2. The long arm of chromosome 14 and the short arm of chromosome X represent 3% and 2% of the haploid genome, respectively, and our observation that more than 16% of the human *elk* probe hybridization was localized to these regions is highly significant ($P < 0.001$). Thus, cytological hybridization localizes the *elk*-1 gene to the region between Xp22.1 and Xp11 and the *elk*-2 gene to region 14q32.3.

In order to identify the cognate gene from which the *elk* cDNA originated, RNAs derived from somatic cell hybrids retaining chromosome X or both X and 14 were tested for expression of *elk*-specific mRNA. In Northern analysis of RNA from human, mouse, and mouse-human hybrid cell lines with *elk*-1 cDNA E2.5 as a probe, human COLO 320 and MOLT-4 cells express a single major *elk*-related transcript ~3.1 kb (Fig. 4A, upper panel, lanes 1 and 2) that is also seen in the hybrid cells of T cell phenotype that retain the human X chromosome and X and 14 (Fig. 4B, lanes 1 and 2). Ribonuclease (RNase) mapping studies on the RNA from these two hybrid cell lines also gave a similar RNase protection pattern (*34*). These results taken together suggest that the cognate gene for the *elk* cDNA is the *elk*-1 locus at Xp11. Preliminary results from a comparison of the restriction maps and chromosomal localization of *elk*-1 and *elk*-2 genomic clones support this conclusion (*35*). Transcripts of the *elk*-2 locus (with *elk*-2–specific probes) were detected in different cells (*36*) indicating that the *elk*-2 locus may represent an active gene.

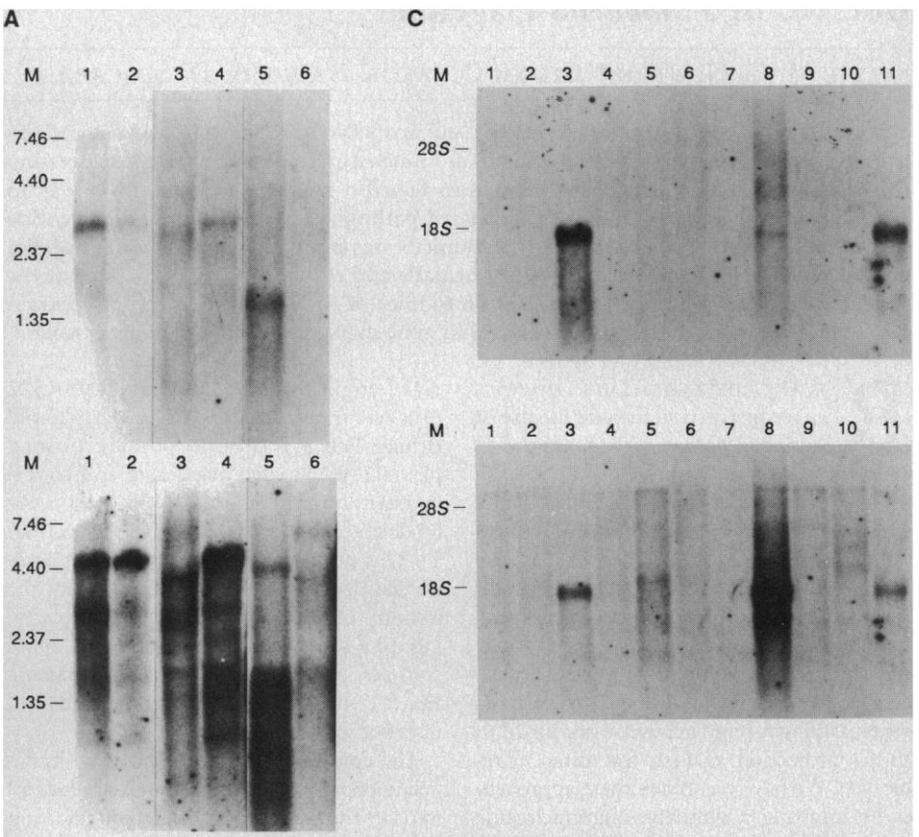

Fig. 4. Expression of *elk* mRNA in human, mouse, and chicken cells. (**A**) Polyadenylated [poly(A)$^+$] RNA from COLO 320 (lane 1), MOLT 4 (lane 2), EL 4 (lane 3), K562 (lane 4), CEF (lane 5), mouse L-M(TK$^-$) cells (lane 6), and BRL RNA ladder were electrophoresed on a 1.2% formaldehyde agarose gel (*18*), transferred to Nytran filter, and hybridized to ^{32}P-labeled 3′ *elk*-1 probe E2.5 as shown in the upper panel. The lower panel depicts the same blot, which was washed and reprobed with a ^{32}P-labeled 5′ *elk*-1 probe, E0.4 (0.4-kb Eco RI fragment from λ 11 clone). RNA sizes were calculated with reference to BRL RNA ladder standard marker, which was detected on reprobing the blot with ^{32}P-labeled λ DNA as described (*45*). (**B**) *elk* expression in hybrid cell lines. Total cytoplasmic RNA (20 μg per lane) was fractionated, transferred to nitrocellulose, and hybridized to the *elk*-1 probe E2.5. Lane 1: T cell hybrid AA3, that retains the human chromosome X but has lost human chromosome 14. Lane 2: T cell hybrid AB3 that retains the human chromosome X and 14q11→14qter. Lane 3: mouse T cell line BW5147. Expression of the human ~3.1-kb RNA correlates with the presence of the human X chromosome. (**C**) Distribution of *elk* transcripts in young and adult mouse tissues. Approximately 10 μg of poly(A)$^+$ RNA from young (5- to 10-day-old mouse) kidney (lane 1), spleen (lane 2), lung (lane 3), thymus (lane 4), liver (lane 5), and adult (5- to 6-week-old mouse) spleen (lane 6), liver (lane 7), testis (lane 8), kidney (lane 9), brain (lane 10), and lung (lane 11) tissues were fractionated. RNA blots were probed with *elk*-1 probe, E2.5 (upper panel) or *elk*-1 probe E0.4 (lower panel) as described. In lane 7, ~5 μg of poly(A)$^+$ RNA was loaded. RNA sizes were determined by comparison with the ribosomal RNA markers (28*S* and 18*S*) as size standards. RNA concentration and integrity of RNA were confirmed by control hybridization of the blot to an actin probe.

Major and minor transcripts of *elk* were observed in COLO 320, MOLT 4, K562, EL 4, L-M(TK$^-$) and chicken embryo fibroblast (CEF) cells (Fig. 4A, upper and lower panels, and Table 1). It remains to be seen whether these different transcripts are generated by alternative splicing and/or polyadenylation of *elk*-1 and/or *elk*-2 or closely related genes. Previously alternative splicing has been observed in the Hu-c-*ets*-1 gene (*15*) and alternative splicing, polyadenylation, and alternative initiation of translation in the human *erg* gene (*18*).

To test whether *elk* expression is associated with specific cell lineages or with cell proliferation, we have studied the expression of *elk* sequences in a variety of proliferating and terminally differentiated tissues from young (5- to 10-day-old) and adult (5- to 6-week-old) mice. *elk* or related sequences were expressed at high levels in both young and adult lung as a major ~1.7-kb species (Fig. 4C, upper panel). Low-level expression of ~4.5-, ~2.8- and ~1.7-kb transcripts was observed in adult testis with a 3′ region *elk*-1 probe (Fig. 4C, upper panel, lane 8). No transcript was detected by a probe from the extreme 3′-untranslated region of *elk*-1, indicating that the major ~1.7-kb mRNA may arise from alternative splicing and/or alternative polyadenylation of *elk* or closely related genes. Hybridization of the Northern blot in Fig. 4C upper panel with a 5′ *elk*-1 probe, showed high expression in adult testis, with a major band of ~1.7 kb and several minor bands (Fig. 4C, lower panel, lane 8). Adult and young lung tissue expressed a single major band of ~1.7 kb. Low-level expression was observed in young liver, (Fig. 4C, lower panel, lane 5) and adult brain (Fig. 4C, lower panel, lane 10).

Thus it appears that, unlike Hu-c-*ets*-1, Hu-*ets*-2 (*37*), and *erg* genes (*38*), *elk* or related sequences are expressed at high levels in both young and adult tissues of nonhematopoietic origin (lung and testis) and moderate levels in lymphoid and myeloid cells. The testis-specific expression suggests that *elk* or related genes may play a role in the differentiation of the male germ cell lineage.

Recently cytogenetic studies of sarcomas in humans have resolved a characteris-

tic chromosomal translocation, t(18;X)(q11:q11), the breakpoint on chromosome X has been assigned clearly at Xp11.2 (*39*). The *elk*-1 gene may be a candidate for involvement in this translocation. *elk*-2 also maps to a chromosome region, 14q32, which may be near characteristic translocation breakpoints. Chromosome band 14q32, the site of the IgH and the putative oncogene TCl-1 (*31, 40*) is frequently rearranged in B and T cell tumors. B cell neoplasias exhibit chromosomal rearrangements within the IgH locus at 14q32.3, whereas in T cell neoplasias with translocation in 14q32, the 14q32 breakpoint usually occurs centromeric to the IgH locus (*31, 41*). The human proto-oncogene AKT-1, a homolog of the viral oncogene v-*akt*, has also been assigned to chromosome band 14q32 centromeric to the IgH locus (*42*). It remains to be seen whether *elk* or related sequences play a role in the pathogenesis of these malignancies.

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Immune Response to Cholera Toxin Epitope Inserted in *Salmonella* Flagellin

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Bacterial flagella are potent immunogens and aromatic-dependent (*aro*) *Salmonella* as live vaccines evoke humoral and cellular immune responses. Such strains expressing epitopes of protective antigens as inserts in flagellin would provide a novel way to vaccinate against diseases caused by unrelated pathogens. A synthetic oligonucleotide specifying an epitope of cholera toxin subunit B was inserted in a *Salmonella* flagellin gene. The chimeric flagellin functioned normally and the epitope was expressed at the flagellar surface. Parenteral administration to mice of an *aroA* flagellin-negative strain of *S. dublin* expressing the chimeric flagellin gene evoked antibody to cholera toxin.

RECENT DEVELOPMENTS IN VACcine construction include the use of synthetic peptides of relevant antigens from various pathogenic microorganisms (*1*); the construction of *Salmonella* strains made nonvirulent by irreversible mutations and therefore safe for use as live vaccines (*2*); and the use of these avirulent strains as carriers of cloned genes that specify foreign proteins to obtain an immune response to heterologous protective antigens (*3*), in much the same way as vaccinia virus carrying foreign genes has been used to confer protection against, for instance, rabies (*4*). We have combined these approaches by inserting a synthetic oligonucleotide into a cloned *Salmonella* flagellin gene; mice given a *Salmonella* live vaccine expressing the recombinant flagellin gene showed an immune response to the inserted epitope.

The flagellin gene used, *H1-d*, determines the phase-1 flagellar antigen, *d*, of *Salmonella muenchen*, a protein of 509 amino acids; its sequence includes a "hypervariable segment" of about 350 bp with no more than 30% amino acid identity to the corresponding sequences in *Salmonella* flagellar antigens *i*, *c*, and *a* (*5, 6*). Epitopes of flagellar antigens *i* (*7*) and *d* (*8*) have been identified in the hypervariable regions of genes *H1-i* of *S. typhimurium* and *H1-d* of *S. muenchen*. A chromosomal Eco RI fragment containing *H1-d*, originally cloned in plasmid pBR322 (*5*), was transferred to pUC19, a plasmid lacking Eco RV sites, to produce plasmid pLS405; the in vitro deletion of an Eco RV fragment gave plasmid pLS408, with a 48-bp deletion in the hypervariable region (Fig. 1). This deletion reduces but does not abolish flagellar function (as inferred from the motility of a flagellin-negative strain carrying pLS408) and removes one or more epitopes of antigen *d* (*8*). The remaining Eco RV site of pLS408 allows the blunt-end insertion of oligonucleotides.

The epitope chosen for expression in flagellin, peptide CTP3, consists of residues 50 to 64 of the B subunit of cholera toxin (*9*); it elicits both polyclonal (*10*) and monoclonal (*11*) antibodies that bind to the peptide and bind and neutralize cholera toxin. A synthetic 45-bp, double-stranded oligonucleotide specifying CTP3, with codon usage corresponding to that in sequenced *Salmonella* flagellin genes (and with a new Cla I site to facilitate analysis) was inserted at the unique Eco RV site of plasmid pLS408 (Fig. 1). We transformed competent cells of a flagellin-deficient *Escherichia coli* strain, CL447 (*12*), and selected for ampicillin-resistant colonies. The oligonucleotide insert improved flagellar function, as judged by the rate of spread in semisolid medium of clones with the insert.

Recombinant plasmids were sequenced by means of a 15-bp primer located 30 bp downstream from the insertion point. Plasmids found to have a single complete copy of the 45-bp sequence in correct orientation

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