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The Complete Sequence and Promoter Activity of the Human A-*raf*-1 Gene (ARAF1)

JONG-EUN LEE,*,† THOMAS W. BECK,* ULRICH BRENNSCHEIDT,‡ LOUIS J. DEGENNARO,§ AND ULF R. RAPP‡,[1]

*Program Resources Inc./Dyncorp. and ‡Laboratory of Viral Carcinogenesis, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, Maryland 21702-1201; †Department of Genetics, George Washington University, Washington, DC; and §Departments of Neurology and Cell Biology, University of Massachusetts Medical School, Worcester, Massachusetts 01655

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The *raf* proto-oncogenes encode cytoplasmic protein serine/threonine kinases, which play a critical role in cell growth and development. One of these, A-*raf*-1 (human gene symbol, ARAF1), which is predominantly expressed in mouse urogenital tissues, has been mapped to an evolutionarily conserved linkage group composed of ARAF1, SYN1, TIMP, and properdin located at human chromosome Xp11.2. We have isolated human genomic DNA clones containing the expressed gene (ARAF1) on the X chromosome and a pseudogene (ARAF2) on chromosome 7p12–q11.21. Analysis of the nucleotide sequence from the ARAF1 genomic clones demonstrated that it consists of 16 exons encoded by minimally 10,776 nucleotides. The major transcriptional start site (+1) was determined by RNase protection and primer extension assays. Promoter activity was confirmed by functional assays using DNA fragments fused to a CAT reporter gene. The ARAF1 minimal promoter, located between nucleotides −59 and +93, has a low G + C content and lacks consensus TATA and Inr sequences but shows sequence similarity at position −1 to the E box that is known to interact with USF and TFII-I transcription factors.

INTRODUCTION

raf-1 was first identified as the cellular homologue of v-*raf*, the transforming gene of 3611 MSV isolated from retroviral transduction experiments (Rapp *et al.*, 1983; Bonner *et al.*, 1985). *raf* proto-oncogenes encode cytoplasmic serine/threonine-specific kinases, which are though to play a critical role in cellular proliferation by mediating mitogen-induced signal transduction from the plasma membrane to the nucleus (Heidecker *et al.*, 1989; Rapp *et al.*, 1988, 1991). A variety of experiments have established that Raf-1 as well as a second intracellular signal transducer Ras function in a signal transduction pathway downstream of growth factor receptors. Briefly, if Ras function in NIH3T3 cells is blocked, which can be accomplished using inactivating antibodies (Smith *et al.*, 1986; Huleihel *et al.*, 1986) or dominant negative versions (Feig and Cooper, 1988), activated Raf-1, unlike growth regulatory protein tyrosine kinases (PTK) and Ras, can bypass the Ras block and transactivate transcription from target genes in the nucleus containing response elements for nuclear oncogenes such as TCF, *jun*, and *ets*, resulting in cell proliferation and ultimately the transformed phenotype (Bruder *et al.*, 1992; Karin and Smeal, 1992; Treisman, 1992; Troppmair *et al.*, 1992; Wasylyk *et al.*, 1989). Blocking of Ras function also demonstrates that Ras is required for receptor-induced phosphorylation activation of Raf (Troppmair *et al.*, 1992). Conversely, if Raf function is blocked, oncogenic versions of Ras will no longer activate transcription from these target genes or transform NIH3T3 cells (Kolch *et al.*, 1991; Bruder *et al.*, 1992). Recently, a protein–protein interaction between Raf and the GTP-bound form of Ras has been described (Moodie *et al.*, 1993; Zhang *et al.*, 1993; Vojtek *et al.*, 1993; Aelst *et al.*, 1993). Moreover, the mitogen-activated protein kinase kinase (MAPKK or MEK) has been identified as a downstream target of Raf kinases (Kyriakis *et al.*, 1992; Dent *et al.*, 1992; Howe *et al.*, 1992).

Homologues of *raf* genes have been identified in multicellular eukaryotes as divergent as higher plants and man (Rapp *et al.*, 1988; Kieber *et al.*, 1993). The *Drosophila* homologue of *raf*-1, D-*raf* or *1* (l) pole hole, has been shown by genetic analysis to be maternally required for cell fate establishment along the anterior–posterior axis of the embryo and zygotically required for somatic cell proliferation and to function downstream of tyrosine kinase receptors such as *torso* and *sevenless* (Mark *et al.*, 1987; Nishida *et al.*, 1988; Ambrosia *et al.*, 1989; Sprenger *et al.*, 1989; Perrimon, 1993). More recently, a downstream target of D-*raf*, designated *Dsor*1, has been identified in *Drosophila* by screening for mutations that

[1] To whom correspondence should be addressed at NIH, NCI-FCRDC, Building 560, Room 21-75, Frederick, MD 21702-1201. Telephone: (301) 846-1309. Fax: (301) 846-1909.

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would suppress D-*raf* mutant phenotypes, and cloning of this gene demonstrated sequence similarity to MAPKK (Tsuda *et al.*, 1993). In *Caenorhabolitis elegans*, vulval differentiation has been shown to require the function of the *lin-45* gene, the *C. elegans* homologue of *raf*-1, which appears to function downstream of *let-60 ras* (Han *et al.*, 1993).

Unlike lower eukaryotes, mammals inherit at least two additional members of the *raf* family of proto-oncogenes, A-*raf* and B-*raf*. These were identified subsequent to *raf*-1 by screening cDNA libraries at reduced stringency, retroviral transduction, or NIH3T3 cell transfection assays of human tumor DNA (Beck *et al.*, 1987; Huebner *et al.*, 1986; Huleihel *et al.*, 1986; Marx *et al.*, 1988; Ikawa *et al.*, 1988). In terms of activation parameters as well as effects on cell physiology, A-Raf functions similar to Raf-1. Briefly, oncogenic activation of both kinases can occur by amino-terminal deletion; A-Raf kinase phosphorylation and activity as measured by activation of MAPKK are induced by mitogens that also regulate Raf-1, and the amino-terminal negative autoregulatory region of A-Raf, like that of Raf-1, functionally blocks Ras signaling (Beck *et al.*, 1987; Huleihel *et al.*, 1986; Grugel *et al.*, submitted).

It is reasonable to expect unique features of A-Raf which may best be studied in tissues in which it is the predominant isozyme. Expression of the 2.6-kb A-*raf* mRNA encoding a 606-amino-acid protein has been determined in mouse and human tissues (Beck *et al.*, 1987; Storm *et al.*, 1990). By Northern analysis, the highest levels of A-*raf* were seen in urogenital tissues, including epididymis, ovary, prostate, kidney, and bladder (Storm *et al.*, 1990). Lower levels were detected in additional tissues, including mouse testes in which expression is restricted to somatic cells, with the highest levels in Leydig cells (Wadewitz *et al.*, 1993). The somatic compartment of the testis is responsible for the production of hormones and is responsive to both steroid and peptide hormones.

A-*raf* belongs to a small group of proto-oncogenes located on the X chromosome. Mapping and genetic linkage analysis in mouse and man have demonstrated that A-*raf* is situated in a gene cluster at Xp11.2 (Huebner *et al.*, 1986) with genes for the neuron-specific phosphoprotein synapsin I (SYN1), the tissue inhibitor of metalloproteinases (TIMP), and the serum glycoprotein properdin (PFC) (Derry and Barnard, 1992). These studies have been confirmed by direct physical mapping of these genes in man and mouse using YACs and pulsed-field gel analyses that showed that they lie within 70 kb of each other in the order of (5′) A-*raf* (3′)–SYN1 (3′)–TIMP–SYN1 (5′)–(3′) PFC (5′) (Derry and Barnard, 1992; Mahtani *et al.*, 1991; Grant and Chapman, 1991; Kirchgessner *et al.*, 1991). This region of the human X chromosome contains at least one gene (TIMP) that is subject to X chromosome inactivation (Brown *et al.*, 1990) and a number of disease genes, including retinitis pigmentosa (RP2), congenital stationary night blindness, and Wiskott–Aldrich syndrome. Among these disease loci, the retinitis pigmentosa (RP2) locus seems to be the closest to the A-*raf* gene locus (Coleman *et al.*, 1990).

Since gene function is determined in part by gene structure, the cloning and characterization of the three known *raf* genes are essential for an understanding of their normal regulation and potential involvement in disease. We have previously described the molecular organization of the human *raf*-1 gene in terms of size (>100 kb), intron/exon structure, nucleotide sequence, and promoter region (Bonner *et al.*, 1986; Beck *et al.*, 1990). In this paper, we present the molecular organization of the human A-*raf*-1 gene (ARAF1), define the minimal DNA sequences necessary for transcription, and present the partial nucleotide sequence of the A-*raf*-2 pseudogene (ARAF2).

MATERIALS AND METHODS

Isolation of genomic DNA clones. The near-full-length A-*raf* cDNA (2.45 kb) from a human T-cell cDNA library (Beck *et al.*, 1987) was subcloned into the *Eco*RI site of pUC18 to generate pART.11. Human placental genomic DNA libraries in EMBL3 (a generous gift of M. Dean) or pWE15 (Stratagene, La Jolla, CA) were plated at 1×10^5 plaque-forming units (22×22-cm Nunc plates) or 2×10^4 colonies (150-mm petri dishes) per plate, and the plaques or colonies were transferred to nitrocellulose or Whatman No. 541 paper, respectively. Hybridizations with ^{32}P-labeled A-*raf* cDNA fragments from pART.11 were carried out at 65°C in 6× SSC, 0.5% SDS, 10× Denhardt's, and 0.2 mg/ml salmon sperm DNA or at 42°C in 50% formamide as described previously (Beck *et al.*, 1990). Phage and bacteria were isolated by 3–4 sequential rounds of screening. Restriction mapping was performed by standard methods (Maniatis *et al.*, 1982).

Subcloning and DNA sequencing. cDNA and genomic DNA fragments were subcloned into pUC19 or pKS vectors using established protocols (Maniatis *et al.*, 1982). Templates for sequencing were generated by forced cloning into M13 or pKS- vectors (Stratagene) and serial deletions using Exonuclease III (Promega, Madison, WI) and sequenced by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using Sequenase (U.S. Biochemicals, Cleveland, OH). The junctions between cloned DNA fragments were confirmed by sequencing asymmetric PCR products from human genomic DNA and/or overlapping genomic clones. Nucleotide sequence alignments and analyses were performed using the software package of the University of Wisconsin Genetics Computer Group at the NCI-FCRF Super Computer Facility (Devereux *et al.*, 1984).

Northern and Southern blot analyses. For Northern blots, 5 μg of total cellular RNA, prepared as described by Storm *et al.* (1990), was denatured with 50% formamide/6% formaldehyde (v/v), separated electrophoretically on 0.7% agarose/6% formaldehyde gels, transferred to nitrocellulose (BA85; Schleicher & Schuell, Inc., Keene, NH), and hybridized with ^{32}P-labeled DNA probes. For Southern blots, 7 μg of Hut 78 genomic DNA was digested with appropriate restriction enzymes, fractionated by electrophoresis through 0.8% agarose gels, transferred to nitrocellulose membranes, and hybridized as described previously (Sithanandam *et al.*, 1989).

RNase protection analyses. The 508-bp fragment containing nucleotide −415 to +93 was subcloned into the *Xho*I/*Xba*I sites of pGEM 7zf and designated pGEM66. This was linearized with *Bgl*II and labeled with T7 RNA polymerase and [^{32}P]CTP, and the labeled RNA product of 209 nucleotides was isolated from a DNA sequencing gel. Hybridizations were carried out in 80% formamide as described previously (Beck *et al.*, 1990) using 20 μg of total human kidney RNA and 200,000 cpm of labeled probe at 42°C overnight and treated with 7.5 units of RNase I (Promega, Madison, WI) at 32°C for 1 h. The products were fractionated on 6% polyacrylamide–8 *M* urea gels.

Primer extension analysis. pARP29 (nucleotides −480 to +300) was 5′ end-labeled at the *Ksp*632I site and digested with *Dde*I, and the 60-nucleotide fragment was isolated as above. Hybridizations were

carried out as described above except that the buffer was 10 m*M* Tris–HCl, pH 8, 1 m*M* EDTA, and 100 m*M* NaCl, and the incubation temperature was 55°C. Primer extensions were performed using MuLV reverse transcriptase (Bethesda Research Labs, Gaithersburg, MD) as described by the manufacturer, and the products were analyzed as described above.

Transfection assays. Cos 7 cells were plated at 5×10^5 cells on 100-mm dishes in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum. Cells were transfected 24 h later with 10 μg of test plasmid DNA by the calcium phosphate method. Cells were washed after 24 h with phosphate-buffered saline (PBS) and replenished with fresh medium. After 48 h, cells were washed twice with PBS, harvested by scraping, lysed in 100-μl 100 m*M* Tris–HCl, pH 7.8, by three cycles of freeze/thaw, clarified by centrifugation in a microfuge (10 min), and assayed for CAT activity using the liquid method (Neumann *et al.*, 1987). Transfection of NIH3T3 cells was carried out as described previously using plasmids linearized with appropriate restriction enzymes and pSV2neo at a molar ratio of 5:1 (test plasmid:pSV2neo) and analyzed 10–14 days post-transfection for transformed foci (Heidecker *et al.*, 1990).

RESULTS

Isolation and characterization of human A-raf genomic clones. Human placental cosmid and lambda phage libraries were screened with the near-full-length (2.45 kb) A-*raf* cDNA. Multiple positive recombinants were identified, isolated, and characterized by restriction mapping and hybridization analyses. One of these, λAR8, hybridized with 5′ and internal cDNA sequences but failed to hybridize with the 3′ untranslated region of the A-*raf* cDNA. However, a genomic clone, 81-1,5,4, originally identified as bearing the 3′ end of the human SYN1 gene (Kirchgessner *et al.*, 1991) hybridized with the A-*raf* 3′ probe, and as illustrated in Fig. 1A, these two genomic clones partially overlapped and were thought to contain the entire ARAF1 gene. Additional genomic clones that were unrelated to the former based on restriction mapping were also identified and therefore were likely to be derived from the A-*raf* pseudogene (ARAF2). The restriction map of one of these, CosAR1, is presented in Fig. 1B.

To determine whether these genomic clones were representative of A-*raf* genes in human DNA, A-*raf* cDNA probes were hybridized to restriction digests of human DNA and the resulting hybridization pattern was compared to the predicted pattern determined from restriction mapping, hybridization, and nucleotide sequence analyses of λ AR8, clone 81-1,5,4, and CosAR1. The results demonstrated that all A-*raf*-hybridizing restriction fragments detected in human DNA were represented in the A-*raf* genomic clones and no major structural alterations had occurred in the cloning of the A-*raf* genes described herein (data not shown).

Nucleotide sequence of the human ARAF1 and ARAF2 genes. Figure 2 shows the nucleotide sequence of the A-*raf* cDNA hybridizing region of λ AR8 and 81-1,5,4 that was compared to the cDNA sequence. The ARAF1 gene consists of 16 exons (Table 1) and contains an intron/exon structure similar to the human *raf*-1 gene with the exception that exons 10 and 11 of *raf*-1 are present without an intervening sequence in the ARAF1 gene. All intron/exon junction sequences conformed to consensus splice junctions except for the exon 13 splice acceptor site, which contained GG as the last two nucleotides instead of the consensus AG (Mount, 1982). In the untranslated exon 1 sequences, there was a single nucleotide insertion at position 28 of the A-*raf* cDNA that was not detected in the ARAF1 gene sequence. However, there were 23 nucleotide mismatches out of the 1215 nucleotides of the reported partial human PKS sequence and the corresponding region of the A-*raf* gene (Mark *et al.*, 1986).

The reported 5′-terminal end of the A-*raf* cDNA corresponds to nucleotide +10 of the ARAF1 gene and the transcriptional initiation site as determined by RNase protection and primer extension assays (see below) to position +1 (Fig. 2). As described previously, on the basis on cDNA sequencing, the 3′ ends of both the ARAF1 and SYN1 genes are in opposite orientation (Kirchgessner *et al.*, 1991) and converge on a single *Eco*RI site. A near consensus poly(A) addition site of CATAAA is located 115 nucleotides downstream of this *Eco*RI site at position 10,891, whereas the reported poly(A) addition site of SYN1 is located 19 nucleotides downstream of this *Eco*RI site, suggesting that the two genes are in part overlapping. These results indicate that the human ARAF1 gene spans at least 10,776 bp.

A computer search of the ARAF1 gene sequence in Fig. 2 for related sequences in the GenBank/EMBL Data Libraries revealed two specific regions of sequence similarity other than to A-*raf* and SYN1. This search revealed the presence of two *Alu*-type repeats at positions −1381 to −1137 and +1124 to +1407. Both sequences showed greatest similarity to the more common *Alu*-S subfamily (Jurka and Smith, 1988).

To definitively establish whether CosAR1 was indeed derived from a pseudogene, a partial nucleotide sequence was obtained. This 219-bp sequence showed 82% sequence identity with the corresponding exons of the ARAF1 gene (Fig. 3) and contained four deletions (3 bp, 18 bp, and two of 1 bp) that resulted in termination codons in all three reading frames. These results establish that the CosAR1 genomic DNA derives from a processed pseudogene that probably corresponds to ARAF2 based on the fact that all non-ARAF1 bands were present in the CosAR1 digest (data not shown).

Mapping of A-raf transcription initiation sites. We used RNase protection and reverse transcription assays to fine map the 5′ end of the A-*raf* gene. For RNase protection mapping, the probe was a uniformly labeled 209-nucleotide copy RNA (*Bgl*II to *Ksp*632I) (Fig. 4A) that was hybridized to total human kidney RNA, treated with RNase, and analyzed on a sequencing gel. As shown in Fig. 4B, major products of approximately 88 nucleotides were detected with kidney RNA but not in the control (*Escherichia coli* tRNA). Primer extension assays using human RNA and an antisense oligonucleotide (nucleotides +99 to +127) resulted in multiple products in the range of 123 to 127 nucleotides; however, additional products presumably resulting from random priming events were also observed (data not shown).

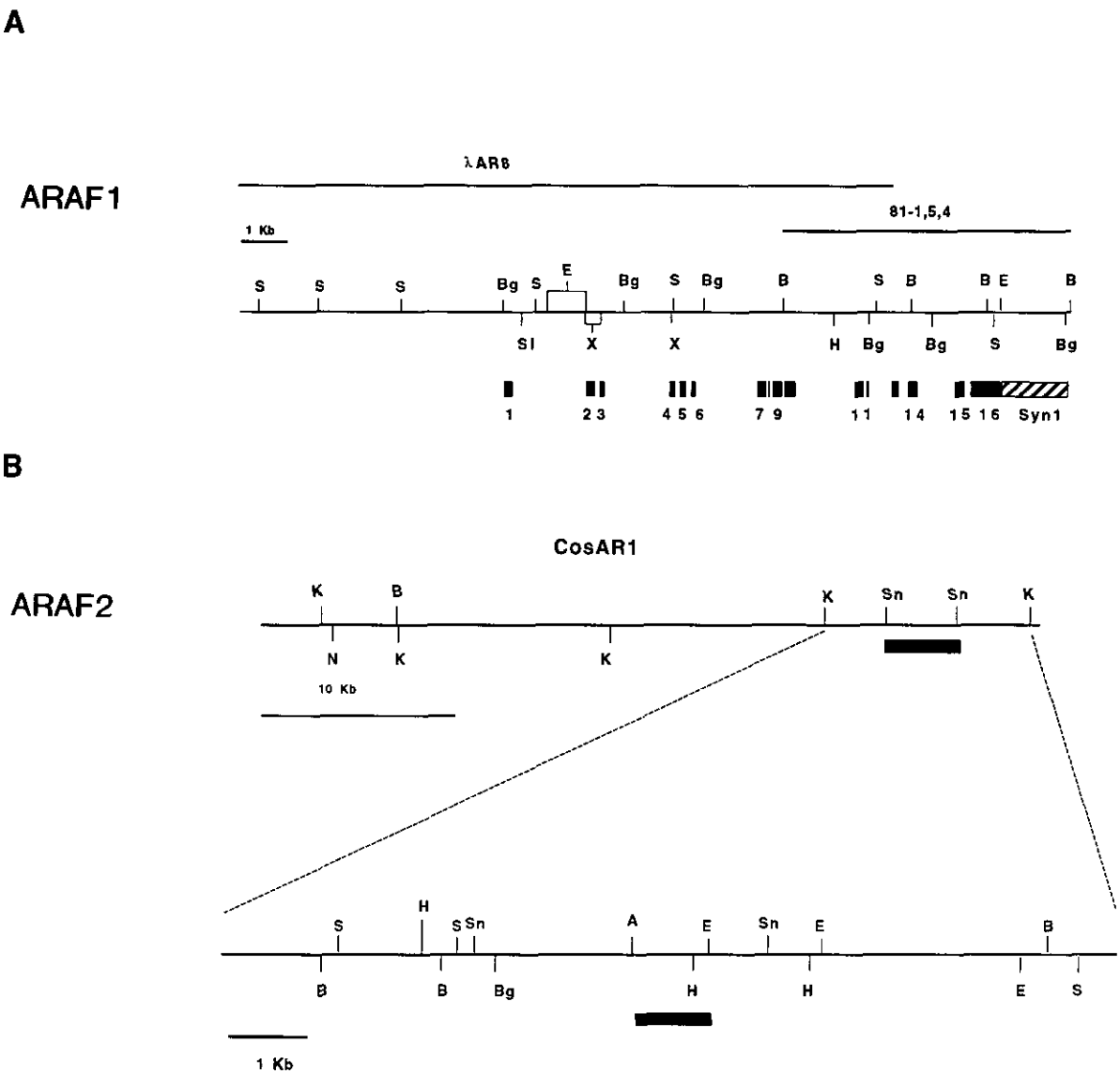

FIG. 1. Restriction map and location of exons of the human ARAF1 proto-oncogene and ARAF2 pseudogene. (**A**) A partial restriction map of the human ARAF1 gene genomic clones λ AR8 and 81-1,5,4 shown with the location of exons 1–16 (black boxes) and the location of exon 13 of the SYN1 gene (hatched box). The restriction sites are *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), *Hin*dIII (H), *Sac*I (S) and *Sal*I (Sl). (**B**) A partial restriction map of the human ARAF2 pseudogene from CosAR1 is shown and the location of A-*raf* hybridizing region is indicated by a black box; restriction sites are as indicated in **A**. and *Apa*I (A), *Kpn*I (K), and *Sna*BI (Sn).

Promoter activity in transient assays and definition of sequences essential to minimal promoter activity. To determine whether this region in fact had promoter activity, we prepared a series of 5′ and 3′ deletions of the 2.0-kb *Pvu*II/*Sac*I fragment fused to the chloramphenicol acetyltransferase gene (CAT) of pUXCAT and tested them directly in Cos 7 cells. The 2.0-kb fragment from the A-*raf* gene (pARCAT37) gave a 25-fold induction over the promoterless CAT vector (pUXCAT), which corresponded to about 50% of the activity obtained with the SV40 early promoter (Fig. 5; data not shown) and was equivalent to the activity of the human *raf*-1 promoter (pCR1CAT) (Beck *et al.*, 1990). In contrast, when placed in the opposite orientation (pARCAT11), little or no promoter activity was detected. Analysis of a series of 3′ deletions indicated that sequences up to the *Ksp*632I site (pARCAT54) retained promoter activity, whereas deletions to the *Bgl*II site (pARCAT51) had lost activity. Analysis of the 5′ deletions in the pARCAT54 background indicated that constructs bearing as little as 59 nucleotides upstream of the putative initiation site (pARCAT69) were fully active. However, deletion of the site to which RNase protection and primer extension assays had mapped the transcrip-

FIG. 2. Nucleotide sequence of the human ARAF1 gene. Shown are the 13550 nucleotides encompassing the A-*raf* gene. The exon nucleotide sequences are capitalized, and corresponding amino acid sequences are indicated underneath. The transcriptional start site corresponding to nucleotide 1 is indicated in bold, and the *Eco*RI site corresponding to the end of the A-*raf* cDNA as well as the SYN1 gene is indicated in bold and underlined.

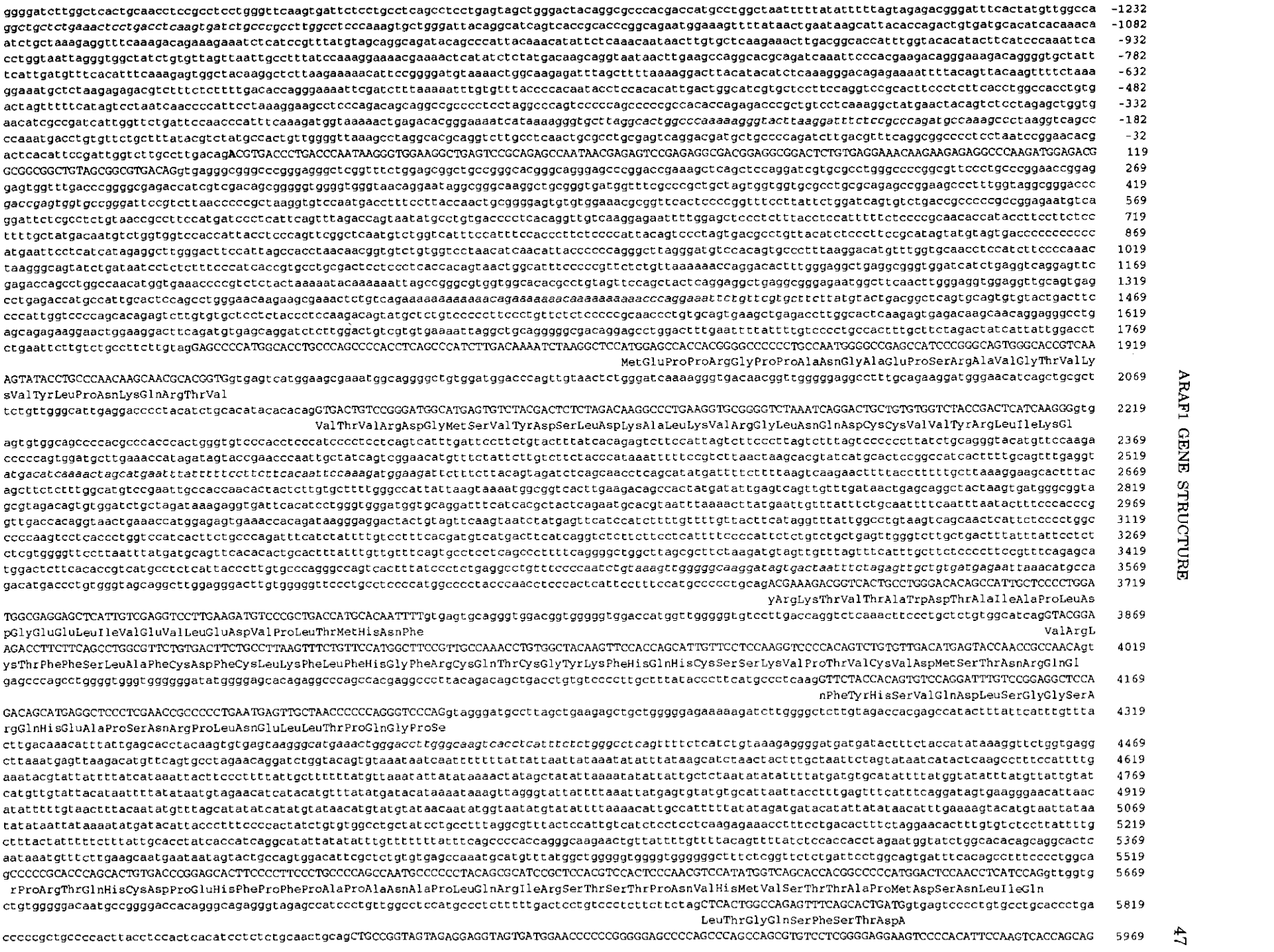

FIG. 2—*Continued*

TABLE 1

Exon–Intron Structure of the Human ARAF1 Gene

Exon	Size	Genomic position	5′ splice donor	Intron	Size	3′ splice acceptor	Codon phase
1	143	1–143	CGTGACAG gtgagg	1	1652	tctgccttcttgtag GAGCCCCA	—
2	255	1796–2050	GCACGGTG gtgagt	2	52	tgcacatacacacag GTGACTGT	O
3	104	2113–2216	ATCAAGGG gtgagt	3	1458	ccatgccccctgcag ACGAAAGA	II
4	103	3675–3777	ACAATTTT gtgagt	4	85	gctctgtggcatcag GTACGGAA	O
5	155	3863–4017	CGCCAACA gtgagc	5	114	cttcatgccctcaag GTTCTACC	II
6	99	4132–4230	GGTCCCAG gtaggg	6	1290	cctttcccctggcag CCCCCGCA	II
7	142	5521–5662	TCATCCAG gttggt	7	104	tccctcttcttctag CTCACTGG	O
8	28	5766–5793	CACTGATG gtgagt	8	74	tctctgcaactgcag CTGCCGGT	I
9	146	5868–6013	AGAAAGTG gtatgc	9	98	ccatctcgcccacag AAGAACCT	O
10	201	6112–6312	GTGCTCAG gtgagg	10	1283	cctccctgcctgcag GAAGACGC	II
11	177	7596–7772	GGCATGGA gtgagc	11	91	ctcatacccccacag CTACCTCC	II
12	47	7864–7910	GTCTAACA gtatct	12	506	tccttgacctggcgg ACATCTTC	I
13	119	8416–8534	TGTGGATG gtgagt	13	236	tctggcctgttgtag GCAGCTGA	O
14	132	8771–8902	GTGACCAG gtgagc	14	853	cccgtctgcccccag ATTATCTT	O
15	135	9756–9890	TCCCCCAG gtgggc	15	308	cccacctgccctcag ATCCTGGC	O
16	578	10199–10776					
Consensus			C(A)AG/GTA(G)AGT			(C or T)$_n$NC(T)AG/G	

tional initiation site (i.e., nucleotide +1) resulted in a complete loss of activity. These results demonstrated that sequences between nucleotides −59 and +93 were essential for promoter activity in transient assays and that the minimal A-*raf* promoter was 151 nucleotides in length.

Transforming activity of an oncogenic version of c-raf-1 under the transcriptional control of the A-raf promoter and primer extension mapping of the transcript. To obtain additional evidence for promoter activity, the 2.0-kb *Pvu*II/*Sac*I fragment from pARCAT37 was fused to the coding sequence of an oncogenic version of the human *raf*-1 cDNA (BXB) such that in the resulting transcript, the splice donor site of exon 1 of A-*raf* would be spliced to the SV40 16S splice acceptor located immediately upstream of the oncogene that included the *raf*-1 poly(A) site downstream of the coding sequence (Fig. 6A). This construct was tested for transforming activity in NIH3T3 cell transformation assays relative to a similar construct containing the human *raf*-1 promoter region (Beck *et al.*, 1990) fused to the same oncogene. The A-*raf* promoter construct (pARP1BXB) directed levels of expression of BXB that resulted in morphological transformation of 60% of the G418-resistant NIH3T3 cell colonies. In the same experiment, the *raf*-1 promoter construct (pRP1BXB) induced transformation of 88% of the colonies.

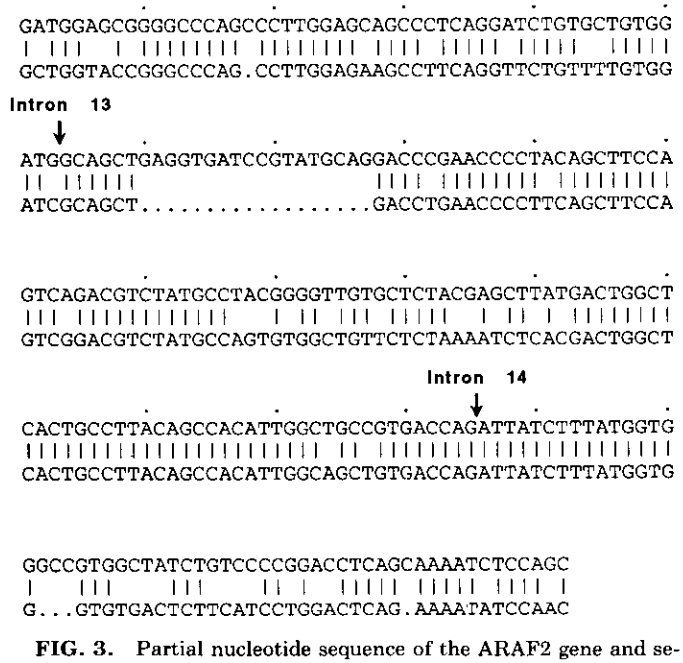

FIG. 3. Partial nucleotide sequence of the ARAF2 gene and sequence comparison with ARAF1 gene. Shown is the partial nucleotide sequence of ARAF2 (lower row) versus the corresponding region of ARAF1 spanning exons 13 to 15 from which introns 13 and 14 sequences have been excised (upper row). Nucleotide identities are indicated by solid vertical lines.

The stably transfected cell lines were further analyzed for the approximate size of the transcription products. Both the A-*raf* and *raf*-1 promoter constructs produced a single RNA species (2.5 and 2.3 kb for pRP1BXB and pARP1BXB, respectively) (Fig. 6B). These results were consistent with proper splicing from the predicted splice donor sequences of each exon 1 to the 16S SV40 splice acceptor preceding the BXB sequences. With the A-*raf* promoter, the RNA size of the product from the pARP1BXB construct, assuming proper splicing, was expected to be shorter than the product of the *raf*-1-driven construct since the *raf*-1 exon 1 is 302 nucleotides and A-*raf* was predicted to be 144 nucleotides. To fine map the transcriptional initiation site in pARP1BXB, primer extension assays were performed using the 59-nucleotide *Dde*I/*Ksp*632I fragment labeled with ^{32}P at the *Ksp*632I site (Fig. 6C), hybridized to RNA from a pool of cells transformed by pARP1BXB or control NIH3T3 cells, and extended with reverse transcriptase. As shown in Fig. 6D, no products were detected with RNA from control NIH3T3 cells or cells expressing BXB from the *raf*-1 promoter, whereas with RNA from NIH3T3 cells transformed with pARP1BXB, a distinct product of 92–93 nucleotides was obtained. The sizes of

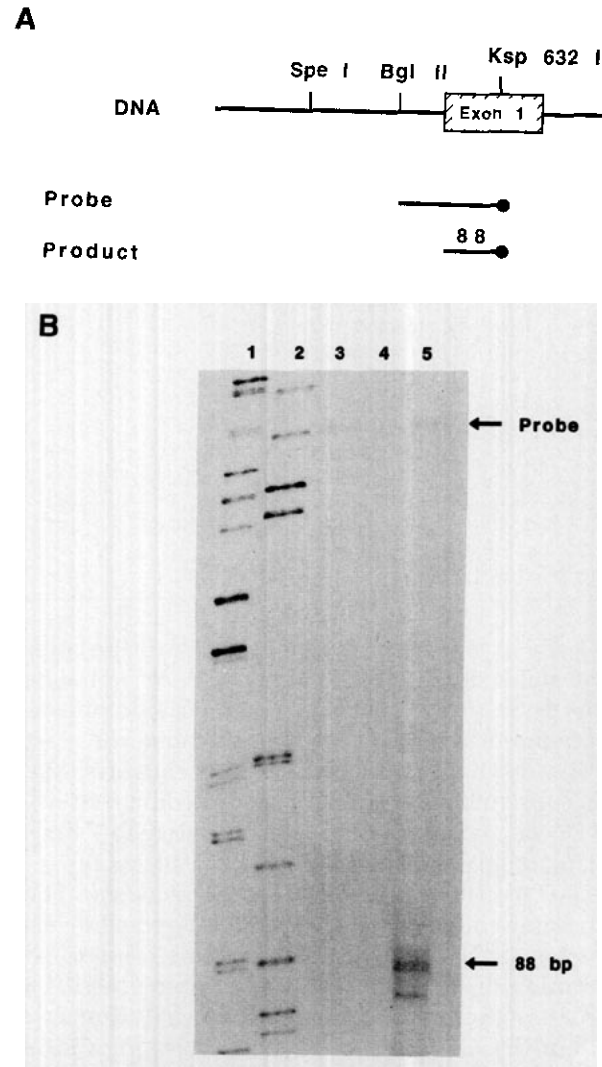

FIG. 4. 5′ end mapping of the human ARAF1 gene. (**A**) Partial restriction map of exon 1 and flanking regions of the A-*raf* gene. The RNase protection probe was a 209-nucleotide copy RNA (nucleotides −71 to +93 and 44 nucleotides of vector sequences) synthesized from the *Bgl*II to *Ksp*632I fragment that had been subcloned into pGEM7zf. (**B**) The RNase protection probe (200,000 cpm) was hybridized to either 20 μg of *E. coli* tRNA (lane 4) or 20 μg of total human kidney RNA and treated with RNase One (Promega), and the products were analyzed on a 6% polyacrylamide–8 *M* urea gel as described under Materials and Methods. The A-*raf*-specific protection products are indicated by an arrow. Lanes **1** and **2** are *Hpa*I and *Hae*III end-labeled fragments of pBR322, and lane **3** is untreated probe alone.

the primer extension products were 4–5 nucleotides longer than those obtained by RNase protection of human kidney RNA. This difference could be accounted for by differential mobility of RNA versus DNA on DNA sequencing gels or breathing of the RNA/RNA hybrid during RNase protection or could represent a true difference between the different sources of RNA. To determine the reason for this slight discrepancy, we compared the RNase protection pattern of RNA from human kidney and NIH3T3 cells expressing pARP1BXB. Both RNA samples gave an identical pattern of RNase-resistant bands (data not shown), indicating that the transcriptional initiation sites in both human kidney and ARP1BXB cells were identical. Therefore, we have assigned the transcriptional initiation site (+1) of the ARAF1 gene as shown in Fig. 2.

Analysis of the ARAF1 promoter region. Figure 7 shows the sequence flanking the start site of transcription of the A-*raf* gene. Computer analysis demonstrated that the region flanking exon 1 has a G+C content of 35–40%, unlike that of the human *raf*-1 promoter (60%). However, the G+C content rapidly increases to greater than 55% in the untranslated sequences of exon 1 and continues into intron 1. This region also has the highest frequency of CpG dinucleotides in the entire ARAF1 gene sequence. In this region, the observed frequency of the dinucleotide CpG was nearly equal to the expected frequency and the ratio of CpG:GpC was 1 as is typical for a HTF island (Bird, 1986). Therefore, although the A-*raf* promoter region is not located in a HTF island, such an island is located immediately downstream.

A search for potential transcription factor binding sites revealed that the region flanking the A-*raf* transcriptional start site lacked consensus CAAT, TATA, and Inr elements. However, as shown in Fig. 7, adjacent to the start site at position −1 is the sequence GACGTG, which shows 5/6 nucleotide identities with the consensus E box (CACGTG), a sequence motif known to interact with the transcription factor USF (Roy *et al.*, 1991). In addition, Fig. 7 illustrates potential binding sites for multiple transcription factors with a known regulatory role.

DISCUSSION

In this paper, we describe the structural and regulatory parts of the ARAF1 gene as well as part of ARAF2 (pseudogene) and present the complete sequence of the human ARAF1 gene. The ARAF1 gene is composed of 16 exons that span 10.8 kb and terminate at or within the SYN1 gene. This makes ARAF1 the smallest of the *raf* genes and its small size may explain why wildtype ARAF1, in contrast to c-*raf*-1 and B-*raf*, has not registered in DNA transfection–transformation assays. In the case of c-*raf*-1 and B-*raf*, the genes are so large (greater than 100 kb) (Beck *et al.*, 1990; Bonner *et al.*, 1986; Sithanandam *et al.*, 1992; Ikawa *et al.*, 1988; Sithanandam, unpublished data) that they are more susceptible to shearing in the process of transfection, which leads to activation of the proto-oncogenes by deletion of 5′ exons that encode negative autoregulatory sequences.

The 5′ end of the human A-*raf* gene was identified by RNase protection and primer extension assays using human RNA, and supporting evidence was obtained using transient and stable transfections of the A-*raf* fragment bearing the RNase protected region fused to a reporter gene. Deletion mapping established that removal of DNA sequences containing the transcriptional start site mapped by RNase protection abolished promoter activity and that the minimal A-*raf* promoter region was located between nucleotides −59 and +93. In addition, using stable transfections, we showed that an oncogenic version of *raf*-1 fused to the A-*raf* promoter region

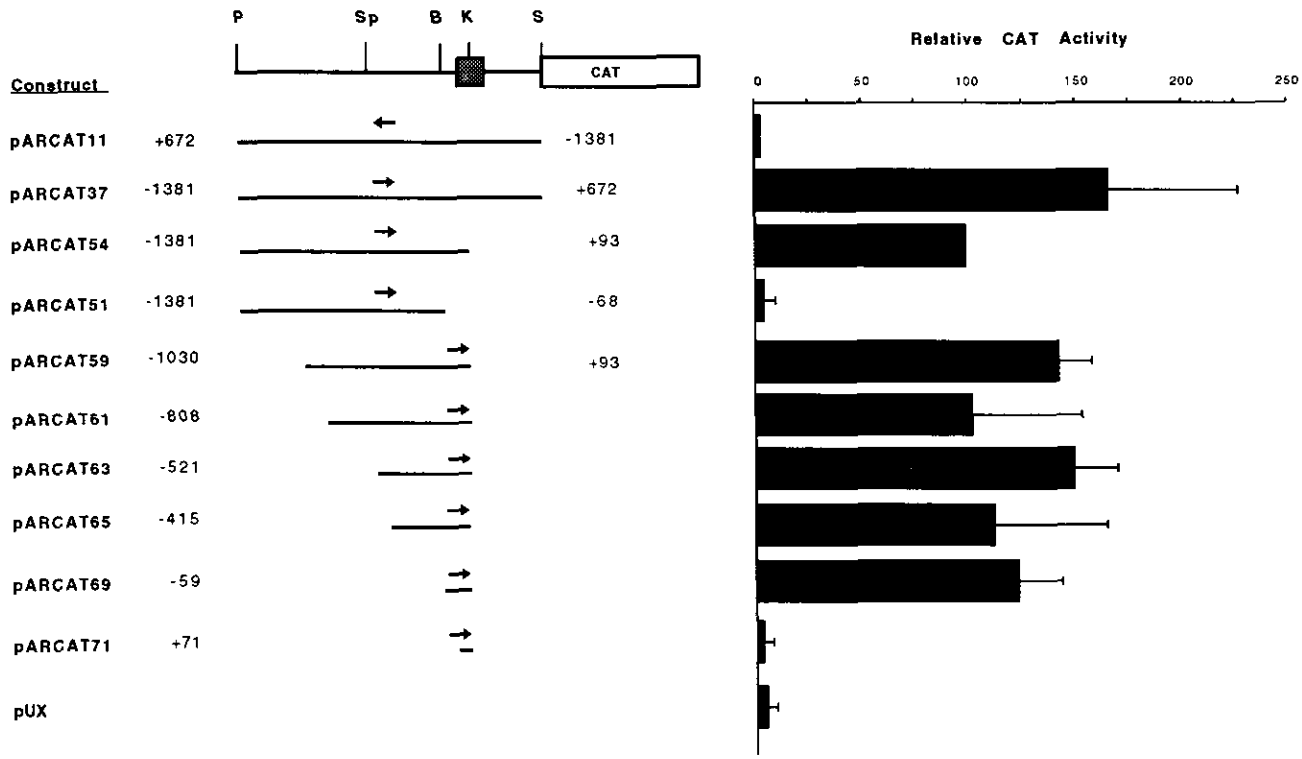

FIG. 5. Transient transfection analysis of the putative A-*raf* promoter region. The 2.0-kb *Pvu*II/*Sac*I fragment from the A-*raf* (nucleotides −1381 to +672) was subcloned into pUXCAT (pUX) in both orientations (indicated by arrows), and various deletions were constructed as indicated. The shaded box represents A-*raf* exon 1 sequences and the locations of restriction sites are indicated: *Pvu*II (P), *Spe*I (Sp), *Bgl*II (B), *Ksp*632I (K), and *Sac*I (S). These were transfected into Cos7 cells and assayed for chloramphenicol acetyltransferase (CAT) activity as described under Materials and Methods. The CAT data are presented as the percentage of construct 54 from at least three independent experiments performed in duplicate with two independent DNA preparations.

(pARP1BXB) was capable of transforming NIH3T3 cells. This construct in NIH3T3 cells resulted in expression of a discrete 2.3-kb transcript whose 5′ end was mapped by primer extension and RNase protection to within 5 nucleotides of the A-*raf* RNA expressed in human kidney and thus represents the 5′ end of the A-*raf* gene. This region has a low G + C content similar to tissue-specific gene promoters such as terminal deoxynucleotidyltransferase (*Tdt*) (Smale and Baltimore, 1989) and unlike the GC-rich human *raf*-1 promoter region (Beck *et al.*, 1990). The A-*raf* promoter lacks consensus CAAT, TATA, and Inr sequence motifs; however, the sequence GACGTG adjacent to the transcriptional start site at position −1 shows 5/6 nucleotide identities with the consensus E box (CACGTG). The E box has been shown to specifically interact with two immunologically related transcription factors, USF and TFII-I (Roy *et al.*, 1991). Interestingly, the latter (TFII-I) is an Inr binding protein that is required for transcriptional initiation *in vitro* from the adenovirus major late core promoter (Roy *et al.*, 1991). Whether the E box is required for A-*raf* promoter activity remains to be determined. The 5′ flanking region includes DNA sequences of potential regulatory significance, including multiple GRE/PRE hormone response elements. This is interesting in light of the preferential expression of A-*raf* transcripts in urogenital tissues, including ovary, testes, epididymis, and kidney, which are known to be steroid hormone responsive.

Analyses of *raf* gene expression in mouse tissues by Northern hybridization has demonstrated that the B-*raf* gene is expressed as multiple tissue-specific transcripts of 13, 10, 4.5, and 2.6 kb, whereas *raf*-1 and A-*raf* are expressed as single transcripts of 3.1 and 2.6 kb, respectively (Storm *et al.*, 1990). Nevertheless, sequence analysis of both *raf*-1 and B-*raf* cDNAs has demonstrated that these genes undergo tissue-specific alternative splicing (Dozier *et al.*, 1991; Eychene *et al.*, 1992), suggesting the possibility that A-*raf* might also express alternative transcripts that are not resolvable on agarose gels. In the quail B-*raf*/c-*Rmil* proto-oncogene, alternative splicing has been demonstrated by the presence of an additional 120-bp exon that alternatively introduces a 40-amino-acid insertion into the hinge region of the B-*raf*/R-*mil* protein between the regulatory and kinase domains of the protein (Eychene *et al.*, 1992). This region corresponds to intron 8 of the ARAF1 gene, which is only 74 nucleotides in length, and bears no consensus splice acceptor sites other than that of exon 9. Moreover, intron 8 contains an inframe termination codon such that a failure to excise intron 8 during processing would result in a RNA encoding a truncated A-*raf* protein. Similar considerations would also apply to the 98-bp intron 9 of ARAF1, making alternative structures in the hinge region of the A-*raf* protein unlikely. The chicken *raf*-1/c-*mil* gene also encodes an alternative exon of 60 bp from intron 10, which introduces 20 amino acids into the chicken *raf*-1/c-*mil* protein (Dozier *et al.*, 1991). Based

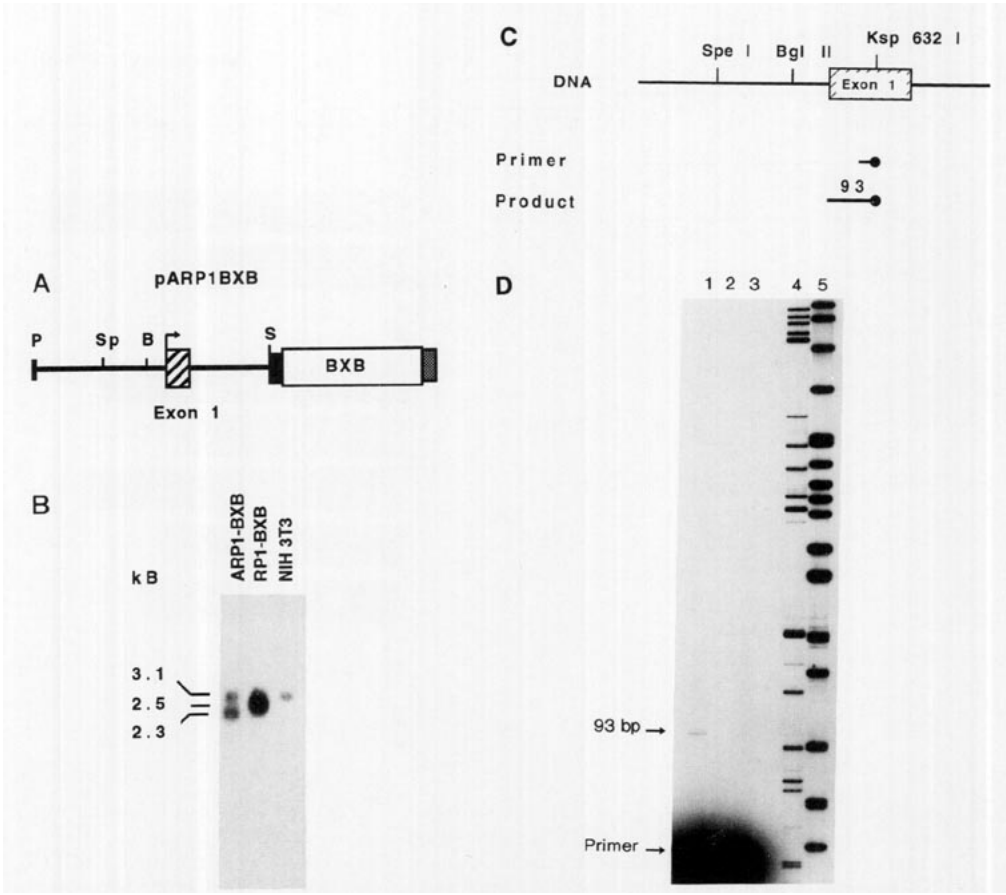

FIG. 6. Analysis of RNA expressed from NIH3T3 cells stably transformed with an oncogenic version of *raf*-1 (BXB) under the transcriptional control of the putative A-*raf* and the *raf*-1 promoter regions. (**A**) Schematic illustration of pARP1BXB. The 2.0-kb *Pvu*II to *Sac*I fragment from the A-*raf* gene was inserted into a construct containing the oncogenic version of the human *raf*-1 cDNA (BXB) that is preceded by a SV40 16S splice acceptor (black box) and followed by a polyadenylation site of the human *raf*-1 gene (hatched box) as described under Materials and Methods. (**B**) Northern blot analysis of RNA transcripts from pARP1BXB, pRP1BXB, and control transfected NIH3T3 cells. NIH3T3 cells (1×10^5) were cotransfected with indicated plasmid and pSV2neo (10/1 μg) per plate. G418-resistant colonies were pooled and total cytoplasmic RNA was isolated. RNA samples (5 μg) were fractionated through 1.6% agarose, transferred to nitrocellulose, and hybridized with a human c-*raf* cDNA fragment labeled with [^{32}P]dCTP. The 3.1-kb band corresponds to the endogenous mouse c-*raf*-1 transcript. (**C**) Partial restriction map of exon 1 and flanking sequence of the A-*raf*-1 gene. The primer extension probe was the 59-bp *Dde*I–*Ksp*632I fragment (nucleotides +35 to +93) indicated with a summary of the resulting products of the primer extension assay. (**D**) Primer extension analysis of pARP1BXB transfected NIH3T3 cells. The primer extension probe was 5′ end-labeled with ^{32}P at the *Ksp*632I site and 100,000 cpm was hybridized to either 10 μg total cytoplasmic RNA from NIH3T3 cells transfected with pARP1BXB (lane **1**), control (lane **2**), or pRP1BXB (lane **3**) and treated with MuLV reverse transcriptase (BRL), and the products were analyzed on a 6% polyacrylamide–8 *M* urea as described under Materials and Methods. The primer and products are indicated by arrows.

on the coordinates from the X-ray structure of PKA (Knighton *et al.*, 1991), these alternate amino acids would be located in a loop between beta-pleated sheets, B1 and B2, which constitute part of the ATP binding domain of the protein. This particular loop region is of variable lengths in different protein kinases (Knighton *et al.*, 1991). The absence of this intron in A-*raf* rules out expression of a similar alternately spliced version of A-*raf*. Whether the alternatively spliced forms of B-*raf* and *raf*-1 represent functionally important differences between the three isozymes is unknown. Based on the gene sequence presented here, we cannot exclude the possibility that other regions of A-*raf* may undergo alternative splicing.

The 3′ end of the A-*raf* gene has not been firmly established due to the absence of a consensus polyadenylation sequence. The predicted size of the transcript based on the 5′ end mapping experiments reported here and the size of the previously published A-*raf* cDNA are in good agreement with mRNA size of 2.6 kb estimated from Northern analysis. This suggests that the A-*raf* polyadenylation site must be within approximately 50–200 nucleotides of the *Eco*RI site. Although no consensus AAUAAA site is located in this region, a variant sequence CAUAAA is located at a position 116 nucleotides downstream of the *Eco*RI site (i.e., nucleotide 10,891). Identical nonconsensus polyadenylation sites have been identified in the thrombospondin 3 and other genes at low frequency (Vos *et al.*, 1992; Wickens and Stephenson; 1984). However, mutational analysis of the consensus AAUAAA sequence suggests that this variant is functionally less efficient in polyadenylation (Wickens, 1990). Since the 3′ end of the SYN1 cDNA also maps to this same *Eco*RI site, the two genes appear to be overlapping in their 3′ termini. The position of A-*raf* in a cluster of genes, at least one of which is known to be X-inacti-

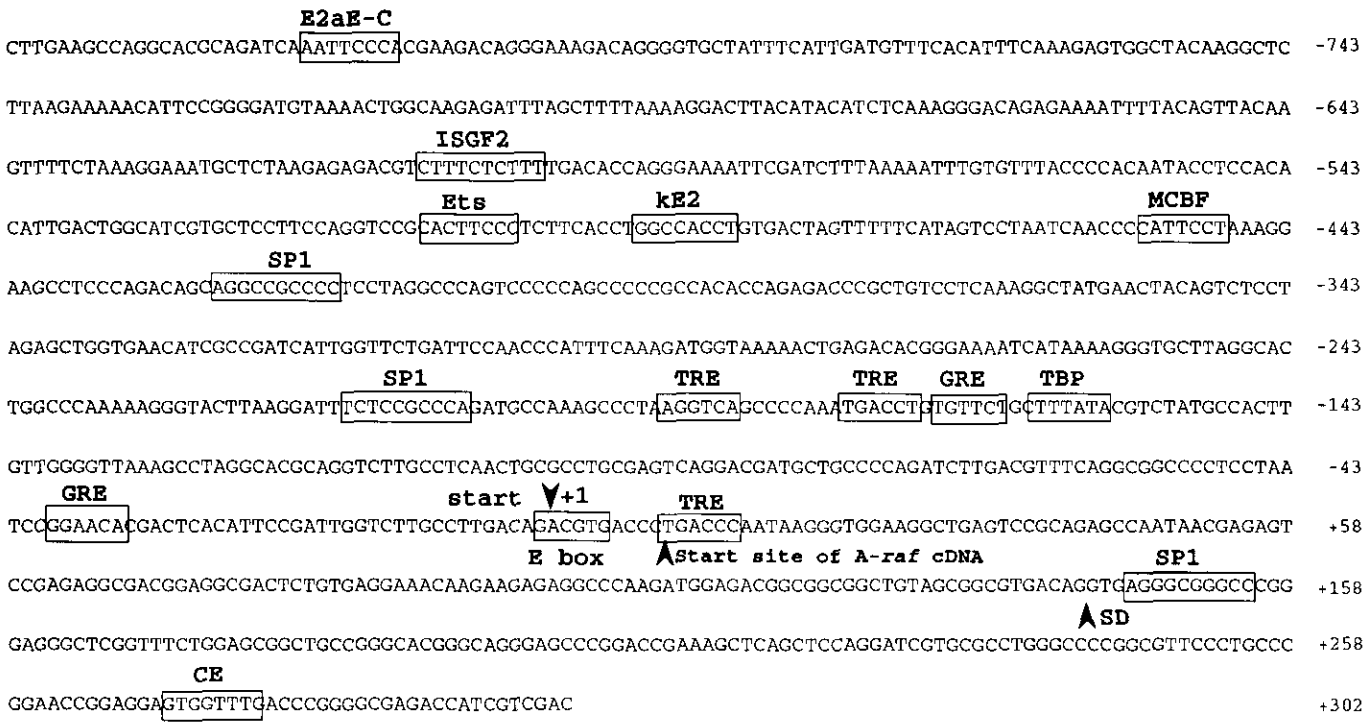

FIG. 7. Nucleotide sequence of the human ARAF1 promoter region. The nucleotide sequence of the ARAF1 promoter region is shown with the major transcriptional initiation site, the A-*raf* cDNA start site, and the splice donor site indicated by arrowheads. Potential transcription factor binding sites (boxes) were identified using MAP (UWGCG) with a transcription factor database derived from published reports (Faisst and Meyer, 1992; Locker and Buzard, 1990). All putative binding sites shown were 100% identical with the consensus sequence with the exception of *Ets* (7/8) and SP1 binding sites (8–9/10).

vated, raises the question of whether this extends to A-*raf*. The fact that there is heterogeneity among the genes in the cluster in terms of promoter-associated methylation sequences that are thought to be important for X inactivation suggests differences in expression. Specifically, the A-*raf* promoter lies in an AT-rich region that is likely to be methylated on CpGs; in contrast, SYN1 is embedded within a HTF island and therefore is likely to be undermethylated. It will be interesting to determine using CA repeat polymorphisms whether A-*raf* is expressed from both the active and the inactive X chromosomes.

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REFERENCES

Aelst, L. V., Barr, M., Marcus, S., Polverino, A., and Wigler, M. (1993). Complex formation between RAS and RAF and other protein kinases. *Proc. Natl. Acad. Sci. USA* **90:** 6213–6217.

Ambrosia, L., Mahowald, A. P., and Perrimon, N. (1989). Requirement of the Drosophila *raf* homologue for *torso* function. *Nature* **342:** 288–291.

Beck, T. W., Huleihel, M., Gunnell, M., Bonner, T. I., and Rapp, U. R. (1987). The complete coding sequence of the human A-*raf*-1 oncogene and transforming activity of a human A-*raf* carrying retrovirus. *Nucleic Acids Res.* **15:** 595–609.

Beck, T. W., Brennscheidt, U., Sithanandam, G., Cleveland, J., and Rapp, U. R. (1990). Molecular organization of the human Raf-1 promoter region. *Mol. Cell. Biol.* **10:** 3325–3333.

Bird, A. P. (1986). CpG-rich islands and the function of DNA methylation. *Nature* **321:** 209–213.

Bonner, T. I., Kerby, S. B., Sutrave, P., Gunnell, M. A., Mark, G. E., and Rapp, U. R. (1985). Structure and biological activity of human homologs of the *raf/mil* oncogene. *Mol. Cell. Biol.* **5:** 1400–1407.

Bonner, T. I., Oppermann, H., Seeburg, P., Kerby, S. B., Gunnell, M. A., Young, A. C., and Rapp, U. R. (1986). The complete coding sequence of the human *raf* oncogene and the corresponding structure of the c-*raf*-1 gene. *Nucleic Acids Res.* **14:** 1009–1015.

Brown, C. J., Flenniken, A. M., Williams, B. R. G., and Willard, H. F. (1990). X chromosome inactivation of the human *TIMP* gene. *Nucleic Acids Res.* **18:** 4191–4195.

Bruder, J. T., Heidecker, G., and Rapp, U. R. (1992). Serum-, TPA-, and Ras-induced expression from Ap-1/Ets-driven promoters requires Raf-1 kinase. *Gene Dev.* **6:** 545–556.

Coleman, M., Bhattacharya, S., Lindsay, S., Wright, A., Jay, M., Litt, M., Craig, I., and Davies, K. (1990). Localization of the microsatellite probe DXS7 and DXS255 on Xp and linkage to X-linked retinitis pigmentosa. *Am. J. Hum. Genet.* **47:** 935–940.

Dent, P., Haser, W., Haystead, T. A. J., Vincent, L. A., Roberts, T. M., and Sturgill, T. W. (1992). Activation of mitogen-activated protein kinase kinase by *v-raf* in NIH3T3 cells *in vitro*. *Science* **257:** 1404–1407.

Derry, J. M., and Barnard, P. J. (1992). Physical linkage of the A-*raf*-1, properdin, synapsin 1, and TIMP genes on the human and mouse X chromosomes. *Genomics* **12:** 632–638.

Devereux, J., Haeberli, P., and Smithies, O. (1984). A comprehensive

set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12:** 387–395.

Dozier, C., Ansieau, S., Ferreira, E., Coll, J., and Stehelin, D. (1991). Alternative splicing of RNAs transcribed from the chicken c-*mil* gene. *Oncogene* **6:** 1307–1311.

Eychene, A., Barnier, J. V., Dezelee, P., Marx, M., Laugier, I. C., and Calothy, G. (1992). Quail neuroretina c-R*mil* (B-*raf*) proto-oncogene cDNAs encode two proteins of 93.5 and 95 kDA resulting from alternative splicing. *Oncogene* **7:** 1315–1323.

Faisst, S., and Meyer, S. (1992). Compilation of vertebrate-encoded transcription factors. *Nucleic Acids Res.* **20:** 3–26.

Feig, L. A., and Cooper, G. M. (1988). Inhibition of NIH3T3 cell proliferation by a mutant *ras* protein with preferential affinity for GDP. *Mol. Cell. Biol.* **8:** 3235–3243.

Grant, S. G., and Chapman, V. M. (1991). Detailed genetic mapping of the A-*raf* proto-oncogene on the mouse X chromosome. *Oncogene* **6:** 397–402.

Han, M., Golden, A., Han, Y., and Sternberg, P. W. (1993). *C. elegans lin-45 raf* gene participates in *let-60 ras*-stimulated vulval differentiation. *Nature* **363:** 133–140.

Heidecker, G., Huleihel, M., Cleveland, J. L., Kolch, W., Lloyd, P., Pawson, T., and Rapp, U. R. (1990). Mutational activation of c-*raf*-1 and definition of the minimal transforming sequence. *Mol. Cell. Biol.* **10:** 2503–2512.

Heidecker, G., Cleveland, J. L., Beck, T., Huleihel, M., Kolch, W., Storm, S. M., and Rapp, U. R. (1989). Role of *raf* and *myc* oncogenes in signal transduction. *In* "Genes and Signal Transduction in Multistage Carcinogenesis" (N. H. Colburn, Ed.), pp. 339–374, Dekker, New York.

Howe, L. R., Leevers, S. J., Gomez, N., Nakielny, S., Cohen, P., and Marshall, C. J. (1992). Activation of MAP kinase pathway by the protein kinase *raf*. *Cell* **7:** 335–342.

Huebner, K., Ar-Rushdi, A., Griffin, C. A., Isobe, M., Kozak, C., Emanuel, B. S., Nagarajan, L., Cleveland, J. L., Bonner, T. I., Goldsborough, M. D., Croce, C. M., and Rapp, U. R. (1986). Actively transcribed genes in the *raf* oncogene group, located on the X chromosome in mouse and human. *Proc. Natl. Acad. Sci. USA* **83:** 3934–3938.

Huleihel, M., Goldsborough, M., Cleveland, J. L., Gunnell, M., Bonner, T. I., and Rapp, U. R. (1986). Characterization of murine A-*raf*, a new oncogene related to the *v-raf* oncogene. *Mol. Cell. Biol.* **6:** 2655–2662.

Ikawa, S., Fukui, M., Ueyama, Y., Tamaoki, N., Yamomoto, T., and Toyoshima, K. (1988). B-*raf*, a new member of the *raf* family, is activated by DNA rearrangement. *Mol. Cell. Biol.* **8:** 2651–2654.

Jurka, J., and Smith, T. (1988). A fundamental division in the *Alu* family of repeated sequences. *Proc. Natl. Acad. Sci. USA* **85:** 4775–4778.

Karin, M., and Smeal, T. (1992). Control of transcription by signal transduction pathways: The beginning of the end. *Trends Biochem. Sci.* **17:** 418–422.

Kieber, J. J., Rothenberg, M., Roman, G., Feldmann, K. A., and Ecker, J. R. (1993). CTR1, a negative regulator of the ethylene response pathway in *Arabidopsis*, encodes a member of the *raf* family of protein kinases. *Cell* **72:** 427–441.

Kirchgessner, C. U., Trofatter, J. A., Mahtani, M. M., Willard, H. F., and DeGennaro, L. J. (1991). A highly polymorphic dinucleotide repeat on the proximal short arm of the human X chromosome: Linkage mapping of the synapsin 1/A-*raf*-1 genes. *Am. J. Hum. Genet.* **49:** 184–191.

Knighton, D. R., Zheng, J. H., Ten-Eyck, L. F., Ashford, V. A., Xuong, N. H., Taylor, S. S., and Sowadski, J. M. (1991). Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science* **253:** 407–420.

Kolch, W., Heidecker, G., Lloyd, P., and Rapp, U. R. (1991). Raf-1 protein kinase is required for growth of induced NIH3T3 cells. *Nature* **349:** 426–429.

Kyriakis, J. M., App, H., Zhang, X., Banerjee, P., Brautigan, D. L., Rapp, U. R., and Avruch, J. (1992). Raf-1 activates MAP kinase-kinase. *Nature* **358:** 417–421.

Locker, J., and Buzard, G. (1990). A dictionary of transcription control sequences. *DNA Sequence* **1:** 3–11.

Mahtani, M. M., Lafreniere, R. G., Kruse, T. A., and Willard, H. F. (1991). An 18-locus linkage map of the pericentromeric region of the human X chromosome: Genetic framework for mapping X-linked disorders. *Genomics* **10:** 849–857.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Mark, G. E., MacIntyre, R. J., Digan, M. E., Ambrosia, L., and Perrimon, N. (1987). Drosophila melanogaster homologs of the *raf* oncogene. *Mol. Cell. Biol.* **7:** 2134–2140.

Mark, G. E., Seeley, T. W., Shows, T. B., and Mountz, J. D. (1986). *pks* a *raf*-related sequence in humans. *Proc. Natl. Acad. Sci. USA* **83:** 6312–6316.

Marx, M., Eychene, A., Laugier, D., Bechade, C., Crisanti, P., Dezelee, P., Pessac, B., and Calothy, G. (1988). A novel oncogene related to c-*mil* is transduced in chicken neuroretina cells induced to proliferate by infection with an avian lymphomatosis virus. *EMBO J.* **7:** 3369–3373.

Moodie, S. A., Willumsen, B. M., Weber, M. J., and Wolfman, A. (1993). Complexes of Ras-GTP with Raf-1 and mitogen-activated protein kinase kinase. *Science* **260:** 1658–1661.

Mount, S. M. (1982). A catalogue of splice junction sequences. *Nucleic Acids Res.* **10:** 459–471.

Neumann, J. R., Morency, C. A., and Russian, K. O. (1987). A novel rapid assay for chloramphenicol acetyltransferase gene expression. *BioTechniques* **5:** 444–447.

Nishida, Y., Hata, M., Ayaki, T., Ryo, H., Yamagata, M., Shimizu, K., and Nishizuka, Y. (1988). Proliferation of both somatic and germ cells is affected in the Drosophila mutants of *raf* protooncogene. *EMBO J.* **7:** 775–781.

Perrimon, N. (1993). The *Torso* receptor protein-tyrosine kinase signaling pathway: An endless story. *Cell* **74:** 219–222.

Rapp, U. R., Goldsborough, M. D., Mark, G. E., Bonner, T. I., Groffen, J., Reynolds, F. H., Jr., and Stephenson, J. R. (1983). Structure and biological activity of *v-raf*, a unique oncogene transduced by a retrovirus. *Proc. Natl. Acad. Sci. USA* **80:** 4218–4222.

Rapp, U. R., Cleveland, J. L., Storm, S. M., Beck, T. W., and Huleihel, M. (1986). Transformation by *raf* and *myc* oncogenes. *In* "Oncogenes and Cancer" (S. A. Aaronson, J. Bishop, T. Sugimura, M. Terada, K. Toyoshima, and P. K. Vogt, Eds.), pp. 6–26, Japan Sci. Press, Tokyo/VNU Press, Tokyo, Japan.

Rapp, U. R., Cleveland, J. L., Bonner, T. I., and Storm, S. M. (1988). The *raf* oncogenes. *In* "The Oncogene Handbook" (E. P. Reddy, A. M. Skalka, and T. Curran, Eds.), pp. 213–253, Elsevier Science Publishers, The Netherlands.

Rapp, U. R. (1991). Role of Raf-1 serine/threonine protein kinase in growth factor signal transduction. *Oncogene* **6:** 495–500.

Roy, A. L., Meisterernst, M., Pognonec, P., and Roeder, R. G. (1991). Cooperative interaction of an initiator-binding transcription initiation factor and the helix-loop-helix activator USF. *Nature* **354:** 245–248.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74:** 5463–5467.

Sithanandam, G., Dean, M., Brennscheidt, U., Beck, T., Gazdar, A., Minna, J. D., Brauch, H., Zbar, B., and Rapp, U. R. (1989). Loss of heterozygosity at the c-*raf* locus in small cell lung carcinoma. *Oncogene* **4:** 451–455.

Sithanandam, G., Druck, T., Cannizzaro, L. A., Leuzzi, G., Huebner, K., and Rapp, U. R. (1992). B-*raf* and a B-*raf* pseudogene are located on 7q in man. *Oncogene* **7:** 795–799.

Smale, S. T., and Baltimore, D. (1989). The "Initiator" as a transcription control element. *Cell* **57:** 103–113.

Smith, M. R., DeGudicibar, S. G., and Stacey, D. W. (1986). Require-

ment for *c-ras* proteins during viral oncogene transformation. *Nature* **320:** 540–543.

Sprenger, F., Stevens, L. M., and Nusslein-Volhard, C. (1989). The Drosophila gene *torso* encodes a putative receptor tyrosine kinase. *Nature* **338:** 478–483.

Storm, S. M., Cleveland, J. L., and Rapp, U. R. (1990). Expression of *raf* family proto-oncogenes in normal mouse tissues. *Oncogene* **5:** 345–351.

Treisman, R. (1992). The serum response element. *Trends Biochem. Sci.* **17:** 423–426.

Troppmair, J., Bruder, J. T., App, H., Cai, H., Liptak, L., Szeberenyi, J., Cooper, G. M., and Rapp, U. R. (1992). Ras controls coupling of growth factor receptors and protein kinase C in the membrane to Raf-1 and B-Raf protein serine kinases in the cytosol. *Oncogene* **7:** 1867–1873.

Tsuda, L., Inoue, Y. H., Yoo, M., Mizuno, M., Hata, M., Lim, Y., Adachi-Yamada, T., Ryo, H., Masamune, Y., and Nishida, Y. (1993). A protein kinase similar to MAP kinase activator acts downstream of the *raf* kinase in Drosophila. *Cell* **72:** 407–414.

Vojtek, A. B., Hollenberg, S. M., and Cooper, J. A. (1993). Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell* **74:** 205–214.

Vos, H. L., Devarayalu, S., Vries, Y., and Bornstein, P. (1992). Thrombospondin 3 (*Thbs3*), a new member of the thrombospondin gene family. *J. Biol. Chem.* **267:** 12192–12196.

Wadewitz, A. G., Winer, M. A., and Wolgemuth, D. J. (1993). Developmental and cell lineage specificity of *raf* family gene expression in mouse testis. *Oncogene* **8:** 1055–1062.

Wasylyk, C., Wasylyk, B., Heidecker, G., Huleihel, M., and Rapp, U. R. (1989). Expression of *raf* oncogenes activates the PEA1 transcription factor motif. *Mol. Cell. Biol.* **9:** 2247–2250.

Wickens, M., and Stephenson, P. (1984). Role of the conserved AAUAAA sequence: Four AAUAAA point mutants prevent messenger RNA 3′ end formation. *Science* **226:** 1045–1051.

Wickens, M. (1990). How the messenger got its tail: Addition of poly(A) in the nucleus. *Trends Biochem. Sci.* **15:** 277–281.

Zhang, X-F., Settleman, J., Kyriakis, J., Takeuchi-Suzuki, E., Elledge, S. J., Marchall, M. S., Bruder, J. T., Rapp, U. R., and Avruch, J. (1993). Normal and oncogenic $p21^{ras}$ proteins bind to the amino-terminal regulatory domain of c-Raf-1. *Nature* **364:** 308–313.