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Jean-Vianney Barnier, Catherine Papin, Georges Calothy, Alain Eychène, Odile Lecoq. The Mouse B- raf Gene Encodes Multiple Protein Isoforms with Tissue-specific Expression. *Journal of Biological Chemistry*, 1995, 270 (40), pp.23381-23389. 10.1074/jbc.270.40.23381 . hal-02525365

HAL Id: hal-02525365

<https://hal.science/hal-02525365>

Submitted on 28 May 2021

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The Mouse *B-raf* Gene Encodes Multiple Protein Isoforms with Tissue-specific Expression*

(Received for publication, March 7, 1995, and in revised form, June 12, 1995)

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The *c-Rmil/B-raf* proto-oncogene is a member of the *millraf* family encoding serine/threonine protein kinases shown to be involved in signal transduction from the membrane to the nucleus. We isolated from a mouse brain library *B-raf* cDNAs containing a previously unidentified 36-base pair alternatively spliced exon located between exons 8 and 9 and, therefore, designated exon 8b. Human and mouse *B-raf* mRNAs also contain the 120-base pair alternatively spliced exon 10 previously described in the avian *c-Rmil* gene. Independent splicing of these two exons, located between the conserved region 2 (CR2) and the catalytic domain (CR3) gives rise to mRNAs potentially encoding four distinct proteins. By using specific sera generated against different portions of B-Raf, we identified at least 10 protein isoforms in adult mouse tissues. Some isoforms, in the range of 69–72 kDa, are not recognized by antisera directed against peptides encoded by exons 1 and 2, indicating the existence of B-Raf proteins with two different NH₂ extremities. The other isoforms, in the range of 79–99 kDa, contain the amino acids encoded by exons 1 and 2, by either or both of the alternatively spliced exons, and, possibly, by another unidentified exon. Analysis of *B-raf* mRNA expression by reverse transcriptase-polymerase chain reaction and immunocharacterization of B-Raf proteins in different tissues of the adult mouse showed a tissue-specific pattern of B-Raf isoforms expression. Interestingly, isoforms containing amino acids encoded by exon 10 are specifically expressed in neural tissues. Taken together, these results suggest that distinct B-Raf proteins could be involved, in a tissue-specific manner, in signal transduction pathways.

Proto-oncogenes of the *millraf* gene family encode serine/threonine protein kinases, which act as signal transducers from membrane-associated receptors to nuclear transcription factors (1, 2). Members of the Raf family play a major role in the regulation of cell proliferation and are also required for the determination of cell fate during embryogenesis (3, 4). The p72/74 Raf-1 protein encoded by the *c-raf/c-mil* gene is ubiquitously expressed in embryonic and adult mouse tissues. This protein has been shown to act downstream of Ras and upstream of MAP kinase kinase (Mek-1) in mammalian and in-

vertebrate cells (1). Raf-1 associates with the GTP-bound Ras protein, the activated form of p21^{ras}. Binding to Ras does not directly activate Raf-1 but rather serves to recruit this protein to the membrane. This step is required for Raf-1 activation by an unknown mechanism and subsequent activation of Mek-1 by Raf-1 (5–11). *A-raf*, the second member of this family (12) encodes a 67.5-kDa protein and is expressed predominantly in the epididymis and ovary (13). Its contribution to the signaling pathway has not yet been investigated.

The third member of this family was identified as the *c-Rmil* gene in avian and *B-raf* in mammalian species (14, 15). Like the Raf-1 protein, the B-Raf protein can be activated by growth factors, such as the epidermal growth factor, the basic fibroblast growth factor, and the nerve growth factor in PC12 cells (16, 17) in a Ras-dependent manner (18, 19) and by granulocyte-macrophage colony-stimulating factor, erythropoietin, and stem cell factor in human and by interleukin-3 in mouse hematopoietic cell lines (20). The B-Raf protein acts downstream of p21^{ras} and upstream of Mek during signal transduction in PC12 cells and appears to be the major Mek kinase in nerve growth factor-stimulated PC12 cells (18, 19, 21, 22) and in brain (23, 24). We also showed that B-Raf interacts *in vivo* with Mek-1 and phosphorylates this protein on both serine 218 and 222 (25). Therefore, B-Raf seems to be homologous to Raf-1 in many aspects of the intracellular signaling pathway.

However, the *c-Rmil/B-raf* gene is expressed in a restricted number of tissues as compared with the ubiquitous *c-raf/c-mil* gene. Thus, high levels of *B-raf/Rmil* mRNA were detected in neural tissues, testes, and fetal membranes by Northern blot analysis (13–15, 26). The number and size of *B-raf* transcripts, ranging between 2.6 and 12 kilobases, depend on the tissue analyzed (13, 14), but the identity, structure, and number of B-Raf proteins have not been unequivocally established. We reported the first identification of the *Rmil/B-raf* gene product in avian neuroretina cells, as a 93.5-kDa protein, which differs from the other Raf proteins by the presence of additional 100 N-terminal amino acids encoded by two exons (27, 28). These two exons are also present in the human *B-raf* gene (29). A *B-raf* carboxyl-terminal specific antiserum revealed the presence of two proteins of 75 and 77 kDa in mouse testis and brain lysates (30). An unidentified protein of 95 kDa was also recognized in mouse brain extracts by an antibody directed against the catalytic domain of *v-raf* (31). Moreover, in the rat pheochromocytoma PC12 cell line, B-Raf proteins were identified either as a single 95-kDa band by tryptic mapping (17) or as two proteins of 67 and 95 kDa (16), depending on the cell clones used. Recently, several proteins with apparent molecular weights ranging from 65,000 to 70,000 and 95,000 to 105,000 were detected in mouse brain extracts with a polyclonal antibody specific to the 12 C-terminal amino acids of B-Raf (23).

We previously reported the presence in some avian *c-Rmil*

* This work was supported by the Centre National de la Recherche Scientifique, the Institut Curie, and a grant from the International Human Frontier Science Program Organization. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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cDNAs of an alternatively spliced exon of 120 bp¹ (exon 10) located upstream of the kinase domain (27) between exons 9 and 11 according to the genomic organization of the coding region of the chicken *c-Rmil/B-raf* gene (28). However, the presence of a similar and of other alternatively spliced exons in mammals remained to be investigated. Therefore, we undertook a detailed analysis of the molecular diversity of mammalian B-Raf proteins and of their tissue distribution in order to gain new insights into the specific implications of this gene in signal transduction.

We describe here the molecular cloning and sequencing of several mouse *B-raf* cDNAs differing by the presence of two alternatively spliced exons. We show that the mouse *B-raf* gene not only contains the 120-bp alternatively spliced exon 10 previously described in avian DNA but also another alternatively spliced exon of 36 bp, located between exons 8 and 9 (exon 8b). The expression pattern of the various *B-raf* transcripts in adult mouse tissues was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR). We also identified at least 10 B-Raf protein isoforms in these tissues by using specific antisera directed against different portions of B-Raf. These isoforms differed by the presence of the alternatively spliced exons 8b and 10, by their NH₂ extremities, and possibly by the presence of other unidentified sequences. Each isoform exhibits a specific pattern of expression in the adult mouse tissues analyzed, those containing amino acids encoded by exon 10 being specifically found in the central nervous system.

EXPERIMENTAL PROCEDURES

Isolation and Characterization of Mouse B-raf cDNAs Containing Alternatively Spliced Exons—*B-raf* cDNA clones were amplified from an adult mouse brain library constructed in the λ Zap vector by PCR using a mouse exon 17-specific antisense oligonucleotide, O₄ (5'-GTAAGTCGACGACAGTTACTCCGTACCTTAC), and a T3 promoter-specific oligonucleotide. A second amplification was done using an exon 14-specific antisense oligonucleotide, O₂ (5'-CATAGTCGACGGTCTCAATGATGTGGAGATGG), and a Bluescript SK primer. PCR products were purified, digested with *Bam*HI and *Sal*I enzymes, and subcloned into the *Bam*HI-*Sal*I sites of the Bluescript vector (Stratagene, La Jolla, CA).

300 ng of poly(A)⁺ RNAs from adult mouse brain or 1 μ g of mRNA from adult mouse tissues were reverse transcribed by priming with random hexamers using avian myeloblastosis virus reverse transcriptase (Amersham Corp.). Locations of *B-raf*-specific primers are indicated in Figs. 1, 2, and 4. Amplifications were done with the following primers, the location of which is indicated in the figures: O₅ (5'-GAGTCGACCAATTCCACAGCCTTCC), O₇ (5'-GAGTCGACGAAATTCACAGAAGTGG), O₆ (5'-GAGTCGACCTTTGATCCTGTAAATCCAC), and O₈ (5'-CTGTGACCTCCATCACCACGAAACC). Southern blots of PCR products, separated on a 2% agarose gel, were done using standard procedures (32) and hybridized with 5'-end-labeled oligonucleotides, specific to the exon 8 sequence (O₅₂₇: 5'-CGACCAGCAGATGAAGATC) or with O₇. The *B-raf* gene was analyzed by PCR using 100 ng of genomic DNA from NIH3T3 cells as a template in a standard reaction, with the same couple of oligonucleotides O₅/O₆ and O₇/O₈. PCR products were cloned in the pUC18 vector and partially sequenced using vector-specific primers.

Total cellular RNA preparations were obtained by the single step method using acidic guanidinium thiocyanate (33). RT-PCR analysis of mouse and human exon 10 were done as for the 36-bp exon 8b, except that the following oligonucleotides were used: O₉ (5'-GAGAC-CAGGGGTTTCGTG), O₁₀ (5'-CATCCGACTTCTGTCTCC), O₁₆ (5'-CATTGATTCCTGTCTTCTG), O₁₇ (5'-CCAGGCTCAAATCAAA-CAC), O₂₃ (5'-CCCCCTGAACCAACTGATG), and O₂₄ (5'-GACTTGATTAGAGACCAAGG). RT-PCR analysis of tissue-specific expression of the different *B-raf* transcripts was performed using standard procedures, as described under "Results," using the oligonucleotides described above and O₁ (5'-CACATTGGATCCGAGATTCAAGTGATGACTGGG). Control of the quality of first strand synthesis was done

using β -actin-specific primers (Stratagene) as described by the manufacturer.

Plasmid Constructions—Plasmids encoding B-Raf proteins were generated by exchanging the sequence of the *c-Rmil* quail cDNA cloned into the following plasmids: pSVL/*c-Rmil* A, pSVL/*c-Rmil* B (27), or pSVL/*c-Rmil* A K483M (kinase-defective mutant) (25), with the homologous mouse fragments containing the alternatively spliced exon 8 and/or exon 10, between the *Sau*I site of exon 7 and the *Sal*I site of exon 12. Expression plasmids, sequenced between these two sites, encode the following B-Raf isoforms: the avian B1 isoform, pSVL/*c-Rmil* A; pCP1, a chimeric B2 isoform containing exon 8b; the avian B3 isoform containing exon 10, designated pSVL/*c-Rmil* B; pCP5, a chimeric B3 isoform; two plasmids encoding proteins in which lysine 483 was substituted to methionine, generating kinase-defective mutants: pSVL/*c-Rmil* A K483M and pCP2, encoding a B1 and a B2 kinase-defective mutant, respectively (25).

Preparation of Immune Sera—The coding sequence of quail *c-Rmil* exons 1 and 2 was subcloned in frame with the sequences of pLC24 bacterial expression vector (34) by PCR amplification using primers specific to the sequence encoding the first amino acids of the *Rmil* protein (5'-GAGGATC-CGATGGCGGCGCTGAGC) and of the last 22 bases of exon 2 (5'-CCAAGCTTACTCTA-GATATATTGACGGTGG). Amplified DNA was generated using pSVL/*c-Rmil* A as template (27). We also cloned the 120 bp of the alternatively spliced exon 10 by the same procedure using primers containing the 15 first bases of exon 10 (5'-TGGGATCCGGCCCCCTTTGAACCAG) and the last 19 nucleotides of exon 10 (5'-CCAAGCTTACTCTGAACACTGGGCCAGG). The template was the pSVL/*c-Rmil* B plasmid, which contains the avian sequence of exon 10 (27). Recombinant plasmids were transferred into *Escherichia coli* SG4044, after sequencing of the amplified inserts, and production of fusion proteins was induced by a 42 °C temperature shift. Bacterial fusion proteins were purified as described (35) and prepared for immunization of rabbits (36).

A rabbit polyclonal antiserum was also raised against the exon 8b-encoded synthetic peptide CEKFLPEVELQDQR conjugated to thyroglobulin (Sigma) through the amino-terminal cysteine residue using maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester (Pierce) as a coupling reagent.

Immunoprecipitation and Western Blotting—Transfection of COS-1 cells was done as described previously (37) with 10 μ g of plasmids and analyzed 48 h later. Transfected cells were washed twice with ice-cold phosphate-buffered saline and lysed in 1.8 ml of cold lysis buffer containing 50 mM Tris-HCl, 1% Triton X-100, 100 mM NaCl, 50 mM NaF, 10 mM Na₄P₂O₇, 5 mM EDTA, 1% aprotinin, 1 mM 4-(2-aminoethyl)-benzenesulfonfylfluoride (Pefabloc SC, Interchim), pH 7.5, and then clarified by centrifugation (13,000 \times g, 15 min). Adult mouse tissues from 3 weeks to 2-month-old animals were dissected, homogenized in lysis buffer, and clarified by ultracentrifugation (100,000 \times g, 30 min).

B-Raf proteins were immunoprecipitated from lysates of COS-1 cells or adult mouse tissues, using 5 μ l of each B-Raf-specific antibody or 5 μ l of rabbit preimmune serum and 50 μ l of Pansorbin (Calbiochem) as a 10% suspension in lysis buffer, and incubated for 2 h in ice. Bacterial pellets were washed four times in lysis buffer, and immunoprecipitated proteins were eluted by boiling for 3 min in 40 μ l of Laemmli sample buffer, migrated on SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride blotting membranes (Immobilon-P, Millipore Corp.). Membranes were rinsed, saturated with blocking solution (20), and incubated with B-Raf antiserum diluted 1:1000 to 1:4000 in blocking solution overnight at 4 °C. Immunostaining was performed with horseradish peroxidase-conjugated anti-rabbit IgG, using the ECLTM Western blotting reagents (Amersham Corp.), according to the manufacturer's instructions.

RESULTS

The Presence of Two Alternatively Spliced Exons in the Mouse B-raf Gene Generates Multiple B-raf Transcripts—A partial sequence of the mouse *B-raf* gene was reported by Miki *et al.* (38), who isolated its catalytic domain by using a cDNA expression cloning system. To identify the *B-raf* gene products, we first cloned *B-raf* cDNAs using PCR-aided amplification of an adult mouse brain cDNA library. PCR-amplified products were subcloned and analyzed by restriction mapping and sequencing. We found that some clones differed by the presence of an additional sequence of 36 bp (Fig. 1A). These 36 nucleotides, which are in frame with the remaining *B-raf* coding sequence, are located between exons 8 and 9, according to the genomic

¹ The abbreviations used are: bp, base pair(s); PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; GMP-PNP, guanylyl-5'-yl imidodiphosphate.

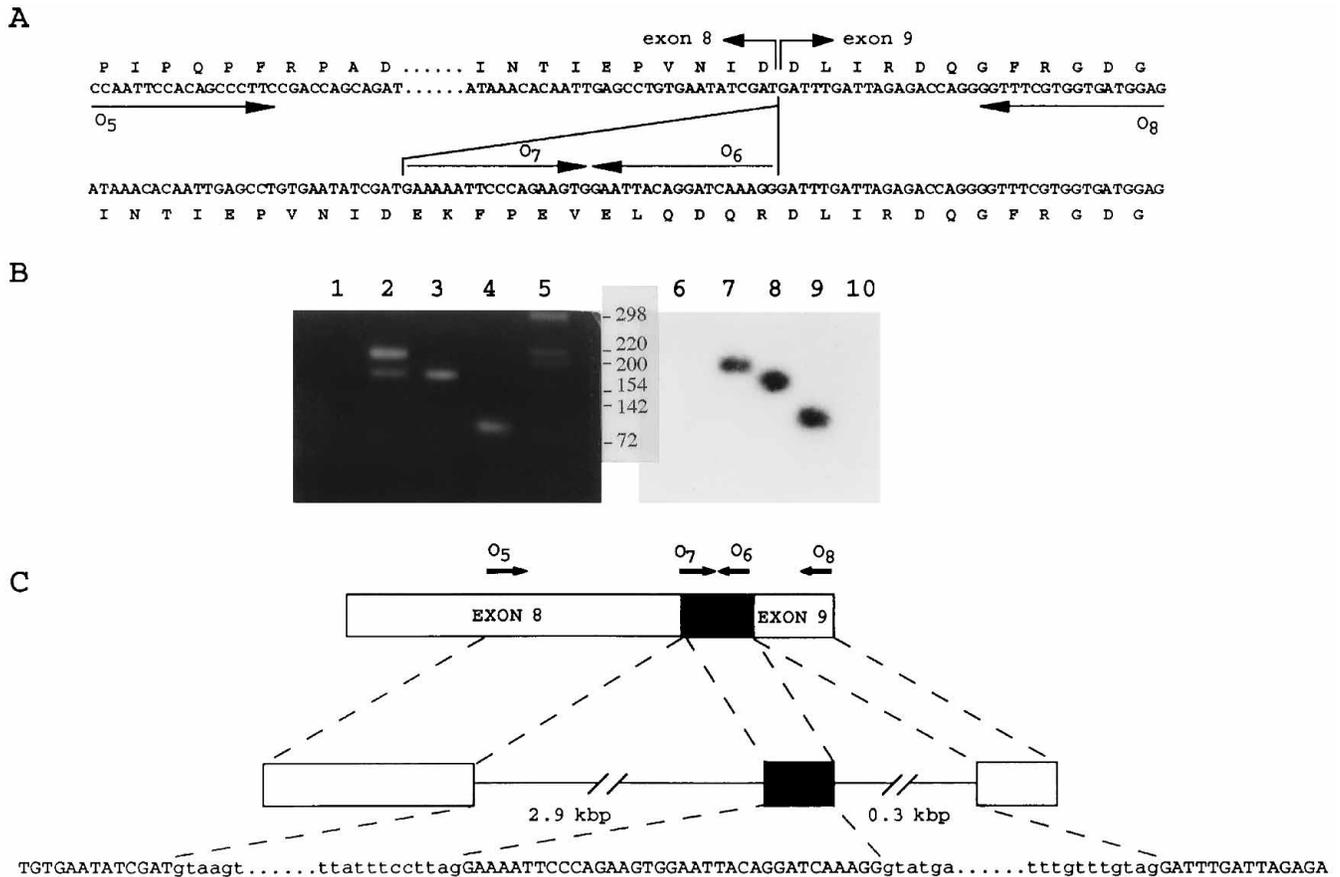


FIG. 1. Identification of a *B-raf* alternatively spliced exon of 36 bp (exon 8b). *A*, partial nucleotide and deduced amino acid sequences of two *B-raf* cDNAs obtained from an adult mouse brain cDNA library by PCR. The locations of exons 8 and 9 are indicated with arrows, according to the genomic organization of the coding portion of the *c-Rmil* gene (28). Oligonucleotides used in PCR amplifications are indicated. *B*, RT-PCR analysis of adult mouse mRNAs (300 ng) amplified with O₅/O₈ (lane 2), with O₅/O₆ (lane 3), and with O₇/O₈ (lane 4). Lane 1, RT-PCR with H₂O as template and O₅/O₈ as primers. Lane 5, size markers are indicated in bp. Lanes 1–5, 2% agarose gel stained with ethidium bromide. Lanes 6–10, PCR products were transferred and hybridized with the labeled O₇ oligonucleotide. Using O₅ and O₈, we amplified two fragments of 157 and 193 nucleotides, which hybridized with an internal exon 8 primer, only the largest hybridizing with O₇. Using O₅ and O₆, we amplified a fragment of 156 bp, which hybridized with O₇. Using O₇ and O₈, we obtained a fragment of 73 bp, which hybridized with O₇. *C*, cDNA structure and genomic analysis of the mouse *B-raf* gene region between exons 8 and 9. 100 ng of DNA from NIH3T3 cells were amplified under standard conditions with O₅/O₆ and O₇/O₈, and PCR fragments were subcloned and sequenced. Exonic sequences are indicated by *capital letters*, and intronic sequences are indicated by *lowercase letters*.

organization of the avian *c-Rmil* gene (28) (Fig. 1A).

We searched for the presence of this sequence in adult mouse brain *B-raf* RNAs using RT-PCR, as described under "Experimental Procedures." We amplified two fragments of 157 and 193 bp with oligonucleotides O₅ and O₈ specific to exons 8 and 9, respectively (Fig. 1B). Both fragments hybridized with an oligonucleotide specific to exon 8 (data not shown), but only the largest one hybridized with the oligonucleotide O₇ specific to the 36-bp insertion. Amplifications with specific primers (O₅/O₆ and O₇/O₈) confirmed these results (Fig. 1B). These observations established the existence of *B-raf* transcripts containing this 36-bp additional sequence.

Since this inserted sequence occurred at the junction between exons 8 and 9, we analyzed the genomic organization of the mouse *B-raf* gene in this region by PCR. NIH3T3 DNA was amplified with either pair of oligonucleotides O₅ and O₆, or O₇ and O₈ (Fig. 1C). Sequencing of amplified DNA fragments showed that this insertion of 36 bp is flanked by 2.9 and 0.3 kilobase pairs of intronic sequences at its 5'- and 3'-ends, respectively. These flanking sequences are in good agreement with the consensus for donor and acceptor splicing sites (39) (Fig. 1C). Taken together, these results show that the 36-bp insertion found in mouse *B-raf* cDNA corresponds to a previously unidentified alternatively spliced exon of the *B-raf* gene.

We previously described an alternatively spliced exon of 120

bp in the avian *c-Rmil* gene, located between exons 9 and 11 (27). We investigated the expression of this exon in adult mouse brain mRNAs, by RT-PCR. Using two oligonucleotides located in the exons 9 (O₉) and 11 (O₁₀), we amplified two fragments of 170 and 290 bp (data not shown). The presence of the 120-bp exon was confirmed by sequencing the 290-bp DNA fragment. Using specific oligonucleotides of this exon 10 sequence (O₂₃/O₁₇), we amplified fragments of the expected size, confirming the presence of this exon in some mouse *B-raf* mRNAs (Fig. 2B). We also searched for the presence of exon 10 in total human *B-raf* RNA purified from a child brain biopsy. Using oligonucleotides O₂₄/O₁₇ and O₂₃/O₁₆, we amplified two fragments of 167 and 174 bp, respectively, in agreement with the presence of an insertion of 120 bp in human brain RNAs (Fig. 2C). When aligned for comparison, the nucleotide sequences of chicken, quail, mouse, and human exons 10 of the *c-Rmil*/*B-raf* genes (27, 28) appeared almost identical (Fig. 2D). The avian sequence differed from the human and mouse sequences by one and three nucleotides, respectively. However, these changes do not modify the deduced amino acid sequence encoded by exon 10, which is, therefore, strictly conserved in the avian and mammalian species (Fig. 2E).

The diversity of *B-raf* mRNAs in the region encompassing exons 8b and 10 was investigated by RT-PCR. Using oligonucleotides O₅ and O₁₀ specific to exons 8 and 11, respectively,

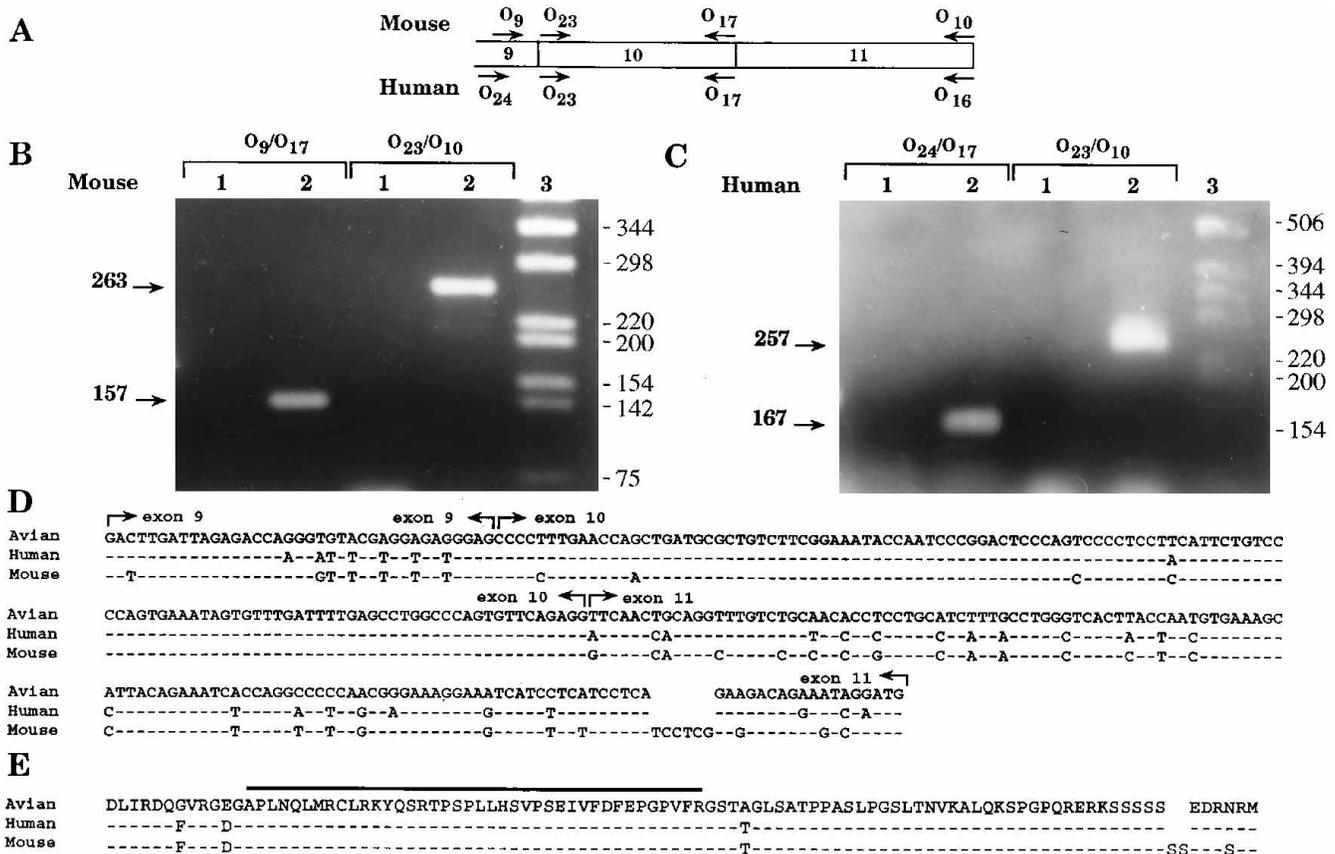


FIG. 2. Identification of the mammalian B-raf alternatively spliced exon 10. A, schematic representation of a partial B-raf exon 10-containing cDNA between exons 9 and 11. Oligonucleotides used for PCR amplification are indicated, in the upper line for mouse cDNA amplification and in the lower line for human cDNA amplification. The genomic organization was deduced from that of the chicken c-Rmil gene (27, 28). B, RT-PCR amplification of adult mouse B-raf mRNAs. 1 μ g of brain mRNA was reverse-transcribed, and 1/20 of the first strand (lane 2) was amplified between O₉ and O₁₇ or O₂₃ and O₁₀. The same primers were used with a control of reverse-transcribed water (lane 1). Size markers are indicated in bp (lane 3). Products were analyzed on a 2% agarose gel. C, RT-PCR amplification of human B-raf mRNA. RT-PCR was done with water (lane 1) or with human mRNAs (lane 2) as template, using O₂₄/O₁₇ or O₂₃/O₁₀ primers. Products were analyzed on a 2% agarose gel. D, comparison of nucleotide sequences of partial cDNAs containing exon 10, from quail, mouse, and human. The chicken coding sequence corresponds to that of the genomic DNA (28). E, comparison of deduced amino acid sequences from avian Rmil and mammalian B-Raf proteins. The sequences of exon 10 are overlined.

and total RNAs from spinal cord as a template, we amplified four distinct fragments of 300, 336, 420, and 456 bp (Fig. 3A). The 420- and 456-bp fragments hybridized with an oligonucleotide specific to exon 10, whereas the 336- and 456-bp fragments hybridized with a labeled oligonucleotide specific to exon 8b (data not shown). Thus, the 300-bp fragment contains exons 8, 9, and 11; the 336-bp fragment contains exons 8, 8b, 9, and 11; the 420-bp fragment contains exons 8, 9, 10, and 11; and the 456-bp fragment contains all five exons (Fig. 3A). These results show that the two alternatively spliced exons are present either together or separately on the same mRNA, suggesting that the B-raf gene is transcribed into at least four distinct mRNAs, designated B1 to B4 (Fig. 3B).

Tissue-specific Distribution of Mouse B-raf Transcripts—Previous reports indicated that the c-Rmil/B-raf gene displays a restricted pattern of expression (13, 14). To delineate the expression patterns of the alternatively spliced exons of the B-raf gene, we analyzed a variety of adult mouse tissues, by RT-PCR using oligonucleotides, the location of which is indicated in Fig. 4A. The number of cycles, which differs between combinations of oligonucleotides, was chosen to optimize signal comparison between the different tissues.

Tissue distribution of B-raf mRNAs was first investigated by using oligonucleotides specific to the catalytic domain, since this domain was shown not to be subjected to alternative splicing (Fig. 4B). We found that B-raf gene expression was rather

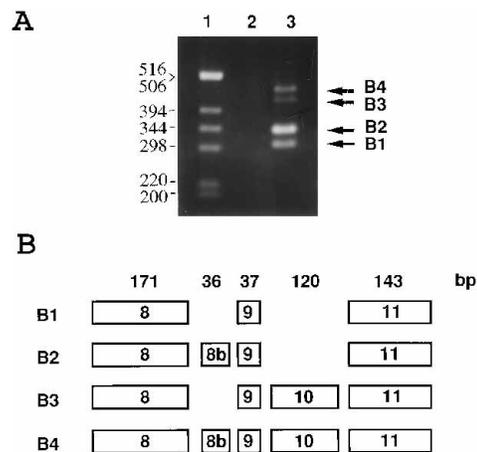


FIG. 3. The B-raf gene potentially encodes four B-Raf isoforms. A, RT-PCR amplification of mouse brain mRNA between exons 8 and 11. 1 μ g of mRNA (lane 3) or water (lane 2) was reverse-transcribed and amplified with O₉/O₁₀ primers and analyzed on a 2% agarose gel. Lane 3, size markers are indicated in bp. Designation of the corresponding isoforms is indicated on the right. B, partial protein structures of the B-Raf isoforms between exons 8 and 11. The size of exons is indicated on the first line.

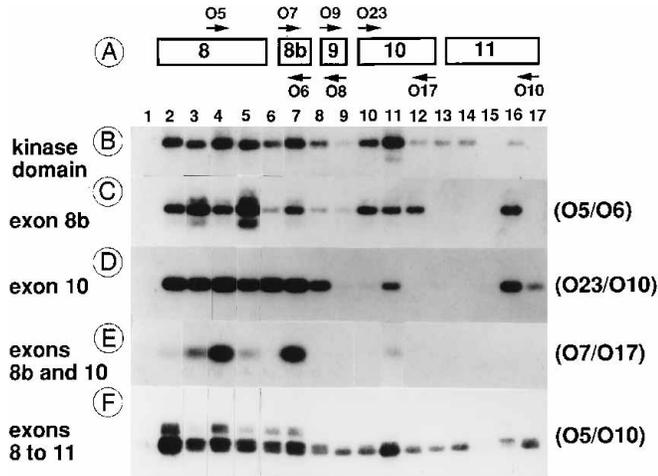


FIG. 4. Analysis of tissue-specific expression of the B-raf transcripts by RT-PCR. The structure of the B-raf transcripts and location of primers used for PCR are illustrated on the first line (A). One μg of total RNA from 14 tissues was reverse-transcribed using random hexanucleotide primers, and $\frac{1}{20}$ of the first strand cDNA was amplified with specific primers indicated on line A. The amount of RNA and quality of the first strand were verified using specific primers of the mouse β -actin gene (data not shown). Adult mouse tissues are as follows: total brain (lane 2), cerebral hemispheres (lane 3), midbrain (lane 4), cerebellum (lane 5), cervical spinal cord (lane 6), dorsal spinal cord (lane 7), eye (lane 8), kidney (lane 9), ovary (lane 10), testis (lane 11), spleen (lane 12), thymus (lane 13), liver (lane 14), muscle (lane 15), heart (lane 16), lung (lane 17). Lane 1 is a control with reverse-transcribed water. Amplifications were done using the following: the kinase domain, O₁/O₄, with 25 cycles and hybridized with labeled O₂ (B); exon 8b, O₇/O₆, with 33 cycles and hybridized with labeled O₇ (C); exon 10, O₂₃/O₁₀, with 33 cycles and hybridized with O₁₇ (D); exons 8b to 10, O₇/O₁₇, with 35 cycles and hybridized with O₉ (E); and exons 8 to 11, O₅/O₁₀, with 30 cycles, and hybridized with O₉ (F).

variable, depending on the tissue. High levels of B-raf transcripts were detected in the nervous system, especially in the midbrain and dorsal spinal cord (lanes 4 and 7). In the total eyes (lane 8), B-raf mRNA is present at an intermediate level, whereas our previous reports showed that this RNA was expressed at a high level in the neuroretina (14). This could be explained by the fact that the neuroretina constitutes only a very small proportion of this organ. High levels of B-raf were also found in gonads, particularly in testes (lane 11), whereas the kidney, spleen, thymus, liver, and heart (lanes 9, 13, 12, 14, and 16) contained intermediate levels of these transcripts. Finally, B-raf was barely detectable in the muscle (lane 15) where 35 cycles of amplification are necessary to detect it (data not shown).

Analysis of the expression of B-raf transcripts containing exon 8b was done following 33 cycles of amplification (Fig. 4C). We found that this exon is widely expressed, but its distribution differs from that of the catalytic domain. Thus, in the central nervous system, exon 8b is highly expressed in the cerebral hemispheres and cerebellum (lanes 3 and 5), whereas its expression is lower in the midbrain and spinal cord (lanes 4 and 6). Interestingly, exon 8b is also found at a moderate level in heart, ovaries, testes, and spleen (lanes 16, 10, 11, and 12). Exon 10 displayed a more restricted pattern of expression (Fig. 4D). It is very abundant in neural tissues (lanes 2–8) in a rather uniform manner and is also detected in the heart and testes (lanes 16 and 11, respectively). Analysis of the B4 transcript expression was performed by amplification between a forward oligonucleotide located in the 5'-end of exon 8b and a reverse primer specific to the 3' extremity of exon 10 (Fig. 4E). Expression of this transcript was restricted to parts of the central nervous system, specifically the mesencephalon and metencephalon (lane 4). We also detected a weak expression in

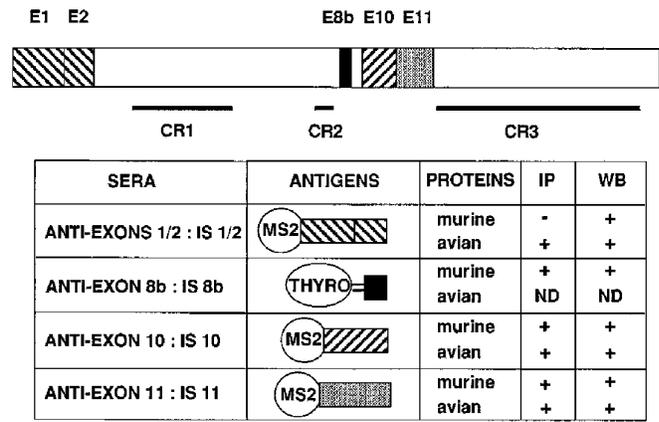


FIG. 5. Characterization of four anti-B-Raf sera. Top, schematic representation of the B-Raf protein, indicating the positions of the Raf family conserved regions, the two alternatively spliced exons (8b and 10), and the location of peptides used for immunizations. Peptides encoded by exons 1 and 2 and exon 10 were fused to a bacterial MSII polymerase and purified. The 12-amino acid peptide encoded by exon 8b was synthesized and coupled to thyroglobulin, as a carrier, before injection to rabbits. Bottom, summary of the designation of sera and the structure of antigens used for immunization. The properties of each serum to immunoprecipitate (IP) and/or recognize the avian or murine (chimeric constructs) Rml/B-Raf proteins by Western blotting (WB) are indicated on the right.

other neural tissues and in the heart.

Expression of the B1 transcript, which does not carry an alternatively spliced exon, was analyzed by RT-PCR (30 cycles) between the forward exon 8-specific primer (O₅) and the reverse exon 11-specific primer (O₁₀) (Fig. 4F). PCR products were hybridized with an oligonucleotide specific to the constitutively expressed exon 9. We obtained the expected four fragments corresponding to B-raf transcripts, which were molecularly characterized. We observed a slight decrease in the amplification efficiency for the large fragments corresponding to the B3 and B4 encoded transcripts. The B1 transcript was widely expressed in adult mouse tissues but was the unique form detected in some tissues such as the kidney, thymus, liver, and lung (lanes 9, 13, 14, and 17), after 30 cycles of amplification. This transcript was the predominant one in testes and ovaries (lanes 10 and 11), whereas its level was lower than that of the B2 form in neural tissues and in the heart (lanes 2–8 and 15).

Characterization of Specific Sera Directed against Exons 1 and 2 and Alternatively Spliced Exons 8b and 10—We previously described an immune serum (IS11) directed against avian Rml amino acids encoded by exon 11 that do not display similarity with other Raf proteins (27). This serum specifically immunoprecipitates and recognizes on Western blots the avian Rml and the mammalian B-Raf proteins. However immunocharacterization of B-Raf proteins with IS11 alone did not allow us to identify the different isoforms. Therefore, we prepared antisera against amino acids encoded by the two alternatively spliced exons 8b and 10, to identify B-Raf proteins containing these exons. These immune sera were designated IS8b and IS10. We also prepared an antiserum directed against the amino acids encoded by exons 1 and 2 of the quail Rml protein in order to confirm the presence of this sequence in B-Raf proteins. The strategy and the structure of the antigenic proteins used to prepare these immune sera are summarized in Fig. 5.

The antisera were tested for their ability to recognize the specific isoforms by immunoprecipitation and Western blotting. Therefore, we transfected COS-1 cells with plasmids encoding the different B-Raf isoforms, and we analyzed their

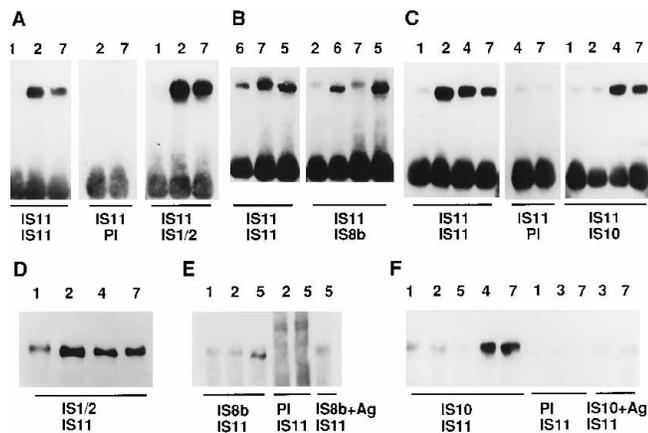


FIG. 6. Characterization of three B-Raf antisera using overexpressed B-Raf isoforms. COS-1 cells were transfected with pSVL vector as control (lanes 1), pSVL/c-Rml A encoding the avian B1 isoform (lanes 2), pSVL/c-Rml A K483M encoding the avian kinase-defective mutant B1 isoform (lanes 3), pSVL/c-Rml B encoding the avian B3 isoform (lanes 4), pCP1 encoding the chimeric B2 isoform (lanes 5), pCP2 encoding the chimeric kinase-defective mutant B2 isoform (lanes 6), and pCP5 encoding the B3 chimeric isoform (lanes 7). Expression of the isoforms in transfected COS-1 cells was controlled by immunoprecipitation of cell lysates followed by a Western blot analysis with the same IS11 antibody. *A*, *B*, and *C*, specific recognition of B-Raf isoforms by the three antisera. COS-1 cells were transfected with the indicated plasmids and immunoprecipitated 48 h later with 5 μ l of IS11 serum. Immunoprecipitates were analyzed by Western blotting as described under "Experimental Procedures" with immune (IS11, 1:4000) or preimmune (PI, 1:4000) IS11 serum (*A*); immune IS11 (1:4000) or immune IS8b (1:1000) (*B*); and immune IS11 (1:4000), preimmune IS10 (1:1000), or immune IS10 (1:1000) (*C*). *D*, *E*, and *F*, immunoprecipitation of B-Raf isoforms. Lysates of transfected COS-1 cells were immunoprecipitated with IS1/2 (10 μ l) (*D*); with IS8b (10 μ l), its preimmune serum (PI 10 μ l), or IS8b preadsorbed with 10 μ g of antigen (*IS8b* + Ag) (*E*); and with IS10, preimmune IS10 (PI), or antibodies preadsorbed with 10 μ g of antigen (*IS10* + Ag) (*F*). Immunoprecipitates were analyzed by Western blotting with IS11 (1:4000).

expression products 48 h later (Fig. 6). We investigated the ability of these sera to recognize B-Raf proteins in a Western blot analysis by immunoprecipitating cell lysates of transfected COS-1 cells with the IS11 serum and subsequently probing the electrophoresed proteins with each of the immune sera (Fig. 6, *A*, *B*, and *C*). The ability of each serum to immunoprecipitate B-Raf proteins was tested by analyzing the precipitated materials by Western blotting and probing with the IS11 serum (Fig. 6, *D*, *E*, and *F*). The properties of these four polyclonal sera are summarized in Fig. 5.

Immunological Characterization of B-Raf Isoform Structures—We analyzed the structure of B-Raf isoforms by immunoprecipitation and Western blotting of extracts from mouse brain and liver, using the four B-Raf-specific sera (Fig. 7). We detected low levels of two proteins of 67 and 69 kDa with the IS11 serum (Figs. 7*A* and 8*A*) but not with the IS1/2 serum (Fig. 7*C*). The 69-kDa protein was also recognized by the IS8b serum. These 67–69-kDa proteins were more easily detectable in Western blots upon longer exposure (Fig. 8, *A* (lane 1), and *B* (lane 1)). The other B-Raf proteins, with apparent molecular weights ranging between 79,000 and 99,000 reacted with both the IS11 and the IS1/2 sera. Taken together, these results indicate that these two 67- and 69-kDa proteins, designated short forms (SF), are B-Raf isoforms lacking most, if not all, amino acids encoded by exons 1 and 2.

In addition, we detected in brain but not in liver extracts proteins that are specifically recognized by the IS8b or by the IS10 serum. This showed that some B-Raf proteins contain the peptides encoded by these alternatively spliced exons, thus confirming the existence of B2 and B3 isoforms (Fig. 7, *D* and

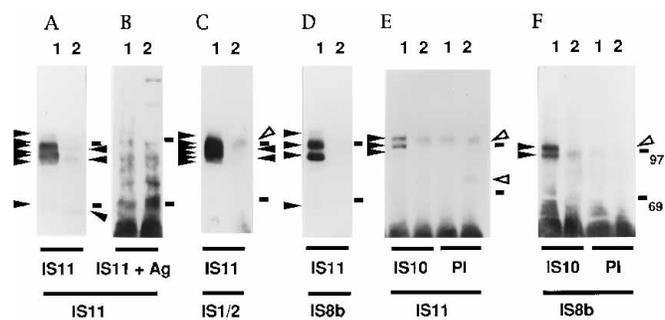


FIG. 7. Characterization of B-Raf isoforms. Brain (lane 1) and liver (lane 2) extracts (400 mg) were immunoprecipitated with IS11 serum (*A*, *C*, and *D*), IS11 preadsorbed with its antigen (*B*), IS10 serum, or its preimmune serum (*E* and *F*). Immunoprecipitates were analyzed on SDS-polyacrylamide gels, transferred on polyvinyl membranes, and probed with IS11 (1:4000) (*A*, *B*, and *E*), IS1/2 (1:1000) (*C*), or IS8b (1:1000) (*D* and *F*) sera. Molecular weights (69,000 and 97,000) are indicated on the right of each gel. Black arrows indicate specific B-Raf proteins; open arrows indicate nonspecific bands. The first lower line indicates the serum used for immunoprecipitation; the second line indicates the serum used for Western blotting.

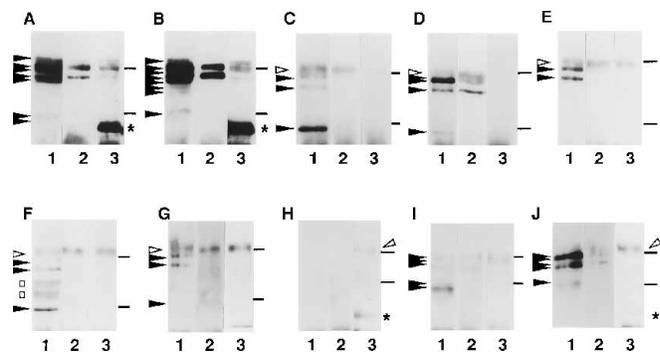


FIG. 8. Tissue-specific expression of B-Raf isoforms. Tissue extracts (brain (*A*), spinal cord (*B*), kidney (*C*), testes (*D*), thymus (*E*), spleen (*F*), liver (*G*), muscle (*H*), heart (*I*), lung (*J*)) (400 mg) were immunoprecipitated with 5 μ l of IS11 serum, and immunoprecipitates were resolved by Western blotting with IS11 (1:4000) (lane 1), with IS8b (1:1000) (lane 2), and with IS10 (1:1000) (lane 3). Exposure time of chemiluminescence was 5 s for neural tissues and 20 s for other tissues. Molecular weights (69,000 and 97,000) are indicated on the right of each panel. Open arrows, asterisks, and open squares indicate nonspecific bands. Dark arrows indicate specific bands.

E). Two proteins with apparent molecular weights of 94,000 and 97,000 were immunoprecipitated with the IS10 serum and recognized by Western blotting with the IS8b serum (Fig. 6*F*). They correspond, therefore, to the B4 isoform. That both proteins were also recognized by the IS1/2 serum (data not shown) rules out the possibility that the difference in their apparent molecular weights could be due to an alternative NH₂ extremity and suggests the existence of an additional alternatively spliced exon(s), as yet unidentified in B-raf cDNAs. It is also possible that they could result from post-translational modifications, such as phosphorylation. These latter isoforms are marked with an asterisk (*).

In summary, the B-raf gene encodes multiple protein isoforms, which differ by the presence of four alternatively expressed regions encoded by exons 8b and 10, the two different NH₂-terminal extremities, and, possibly, an unidentified alternatively spliced exon(s). Since these four alternative structures would potentially generate 16 isoforms, we obviously did not detect all possible B-Raf combinations. For example, the short NH₂ extremity was never found associated with the presence of exon 10. Our analysis of B-Raf proteins in liver, in brain, and in other adult mouse tissues (see below) allowed us to detect only 10 distinct B-Raf isoforms. Their designations, apparent mo-

TABLE I
Characterization of *B-Raf* isoforms

The designation and apparent molecular mass of each isoform are given in the first two columns. Immunological characterization with the different sera was deduced from experiments in transfected COS-1 cells or in mouse tissues. E, exon.

Isoform	Apparent molecular mass	Anti-E1/2	Anti-E8b	Anti-E10	Anti-E11
	<i>kDa</i>				
SF1	67	—	—	—	+
SF2	69	—	+	—	+
B1	79	+	—	—	+
B2	82	+	+	—	+
B1*	86	+	—	—	+
B2*	88	+	+	—	+
B3	90	+	—	+	+
B3*	94	+	—	+	+
B4	96	+	+	+	+
B4*	99	+	+	+	+

lecular weights, and immunologically deduced organizations are summarized in Table I.

Expression of *B-Raf* Isoforms in Adult Mouse Tissues—We immunoprecipitated B-Raf proteins from 10 adult mouse tissues with the IS11 serum and analyzed them by Western blotting with each of the four specific immune sera (Fig. 8 and Table II). The highest levels of B-Raf protein expression were detected in the brain (Fig. 8A) and spinal cord (Fig. 8B), as compared with other tissues. Brain regions presented the most complex pattern of expression. They are the only tissues expressing all four alternatively spliced regions. Because of the number of detectable bands, it was difficult to precisely distinguish these proteins. In brain and spinal cord, we could detect seven and nine isoforms, respectively, with the IS11 serum, and four isoforms with the IS10 serum. The main differences between these two tissues were that the exon 8b-containing isoforms were more abundant in the spinal cord, and that the B1 and B1* forms were detected only in the spinal cord.

In other tissues, B-Raf proteins were less abundant and their expression pattern was less complex than in neural tissues. In kidney (Fig. 8C), we detected three isoforms, the major one being the short form SF1 of 67 kDa, and the B1 and B1* forms were weakly detected. Thus, the kidney does not appear to express the two alternatively spliced exons 8b and 10. Among the 10 tissues tested, the liver (Fig. 8G) had nearly the same isoform pattern of expression as the kidney, but the SF1 form was less expressed and the B1 and B1* forms were more abundant. Both B1 and B1* isoforms were moderately expressed in the thymus, which appears to be the unique tissue to express only two isoforms.

All other tissues contained B-Raf isoforms carrying the polypeptide encoded by exon 8b. In the spleen (Fig. 8F), we detected three isoforms, the most abundant being a short form of 69 kDa, which was also recognized by the IS8b serum. The two other isoforms were the B1 and B1* proteins. In the testes (Fig. 8D), a weak expression of the SF1 form was also observed. We characterized two isoforms recognized with the IS8b serum, corresponding to the B2 and B2* forms. The B1* protein was also present in this tissue.

The lung (Fig. 8J) expressed moderate levels of B1, B1*, B2, and B2* and also weak levels of the SF1 isoform. The heart (Fig. 8I) is the only non-neural tissue to express a detectable amount of exon 10-containing proteins and also the only one that did not express the unidentified structure (*). However, the major isoform detected in this tissue was the SF1 form. We detected two proteins with the IS10 serum, one of which reacted also with the IS8b serum; thus, they corresponded to the B3 and B4 isoforms, respectively. Interestingly, we could not detect B-Raf proteins in the muscle. Taken together, our results on the expression pattern of the various B-Raf isoforms

are in agreement with those obtained by RT-PCR analysis.

DISCUSSION

Molecular analysis of *B-raf* transcripts and immunocharacterization of B-Raf proteins allowed us to identify and to characterize at least 10 B-Raf isoforms in adult mouse tissues, each of them exhibiting a particular pattern of distribution. We showed the presence of two alternatively spliced exons and proposed the existence of an additional one. This demonstrates the high degree of complexity in the structure of *B-raf* gene products, as compared with other protein kinases. Our results also suggest the existence of tissue-specific regulation of alternative splicing and selection of B-Raf isoforms in adult mouse tissues.

Structure of the *B-Raf* Isoforms—We provide evidence that two proteins with an average molecular weight of 67,000–69,000, which are recognized by B-Raf-specific immune sera, are indeed B-Raf proteins and differ from the longer forms by their amino-terminal extremities. These short forms are specifically recognized by some anti-B-Raf antibodies but not by the IS1/2 serum. We could exclude the possibility that they originate from the long isoforms by proteolytic cleavage or degradation since we did not detect them in COS-1 cells transfected with full-length cDNAs. It is possible that expression of the *B-raf* gene could be regulated by at least two alternative promoters, each one controlling the expression of one type of B-Raf protein. Alternatively, it may be that the two different NH₂ extremities are generated by an alternative splicing mechanism.

It is likely that the 67-kDa B-Raf protein identified in PC12 cells (16, 40) and in brain extracts (23) corresponds to one of the short forms described in this study. However, we did not detect this short form in several PC12 cell line clones used in our laboratory, as also reported by Stephens (17). This discrepancy may be due to the PC12 cell clones used. Interestingly, these short forms also present a restricted pattern of distribution and appear to represent the major isoform in some tissues, such as the kidney.

Indirect evidence that the 67/69-kDa short forms possess a functional kinase domain is provided by a convergent set of data. In some PC12 and hematopoietic cell lines (16, 20) in which activation of B-Raf was studied, an increased phosphorylation and a relative retardation in gel electrophoretic mobility of the p67/p69 B-Raf protein were detected after cytokine activation of these cells. Because of the presence of the 95-kDa protein in these cells, it was not possible to conclude whether p67/p69 possesses a kinase activity or whether it is a substrate of p95^{B-raf}. We recently showed that a short isoform was the only B-Raf protein detected in Jurkat cells and that this protein possesses an intrinsic kinase activity, which increased after

TABLE II
Expression of B-Raf isoforms in adult mouse tissues

Expression of B-Raf isoforms was deduced from Western blotting experiments described in Figs 7 and 8. B, brain; SC, spinal cord; KI, kidney; TE, testes; TH, thymus; SP, spleen; LI, liver; MU, muscle; HE, heart; LU, lungs. Quantification was done by visual estimation. Blank, not detected; +, weak; ++, moderate; +++, abundant; +++++, very abundant.

Protein	B	SC	KI	TE	TH	SP	LI	MU	HE	LU
SF1	+		++++	+			+		++	+
SF2	++	+				++			+	
B1		++	+		++	+	++			++
B2		++		++					+	+
B1*	+++	+++	+	++	++	+	++			++
B2*	++++	++++		++						++
B3	+++	+++							+	
B3*	++++	++++								
B4	++++	++++							+	
B4*	++	++								

cell stimulation (20). Moodie *et al.* also reported that different B-Raf proteins, with molecular weights ranging between 65,000 and 105,000, associate with immobilized p21^{ras}-GMP-PNP (23). Taken together, these results suggest that the short B-Raf isoforms interact directly with activated p21^{ras} protein and that this interaction apparently does not require the presence of the first two coding exons. Interestingly, the B-Raf short forms, which apparently do not contain sequences encoded by exons 1 and 2, have a structure similar to that of the A-Raf- and Raf-1-related proteins. Thus, their specific role in signal transduction remains to be elucidated.

We also identified two alternatively spliced exons, 8b and 10, encoding 12 and 40 amino acids, respectively, which do not present similarity with known protein sequences. Using specific antibodies IS8b and IS10, we identified 5 and 4 isoforms, respectively, containing these sequences and showed that both inserts can be associated on the same protein.

The 36-bp exon 8b, between exons 8 and 9, is located at a position homologous to that of the 7a alternatively spliced exon identified in the *c-mil/c-raf* gene (41). This 60-bp *c-mil/c-raf* exon encodes 20 amino acids, which are not similar to those encoded by exon 8b of the B-raf gene. Interestingly, expression of the 7a *c-mil/c-raf* exon is restricted to muscle and, to a lesser extent, to brain (42) suggesting that the tissue-specific exons 7a and 8b could have a specific function in signal transduction.

Exon 10, the amino acid sequence of which is conserved between avian and mammalian species, displays the more restricted pattern of expression since it was found expressed at a high level only in the central nervous system and, to a lesser extent, in heart and testes, as determined by RT-PCR. Exon 10-containing isoforms were detected with the IS10 serum in neural tissues and weakly in the heart. We did not detect exon 10-containing proteins in testes, probably because of the lower sensitivity of the immunological assay. It might be that these isoforms are expressed specifically in neural structures of the heart, which would strongly suggest that alternative splicing of this exon takes place preferentially in neural cells.

Exons 8b and 10 are located in the same region, between the CR2 and the CR3 domains of the B-Raf proteins. They are separated only by the 37 nucleotides of exon 9. This region presents a high polymorphism in B-Raf and also, to a lower extent, in Raf-1 proteins and could, therefore, correspond to a variable region in the *raf* gene family, as reported for calmodulin kinases (43). Our immunological data suggest that B-Raf proteins contain at least another alternatively expressed structure, which could either correspond to a post-translational modification of the protein or to a sequence encoded by an unidentified alternatively spliced exon. Extensive molecular analysis of B-raf transcripts should help to confirm this hypothesis.

Tissue Distribution of B-Raf Isoforms—Our molecular and

immunological results show that the B-raf gene is highly expressed in neural tissues, such as the brain and spinal cord. In these tissues, the long forms are predominant. We did not observe a significant difference in B-raf expression in different regions of the brain, in contrast to the report of Storm *et al.* (13). As observed by RT-PCR, the two alternatively spliced exons 8b and 10 are highly expressed in brain, and exon 10 appears to be neurospecific. A more precise analysis of B-raf expression in the heart and in the central nervous system could confirm this hypothesis. The higher complexity in the expression pattern of B-Raf isoforms was also found in neural tissues. We detected eight and nine isoforms in the brain and spinal cord, respectively. Similar results were obtained by analyzing adult rat brain extracts (data not shown).

In other tissues, B-raf expression is also complex but quantitatively less important. We observed a relatively elevated level of B-raf transcripts and B-Raf proteins in testes as also reported by Storm *et al.* (13), but this expression is weaker than in neural tissues. Our results establish that other tissues express relatively weak amounts of B-raf transcripts and proteins and that expression of this gene is barely detectable in muscle. Moreover, we showed that the pattern of isoform expression is specific to each tissue.

B-Raf Isoforms and Signal Transduction—The CR1 and CR3 domains of the Raf family proteins have been shown to interact with cellular proteins, such as Ras-GTP, the 14-3-3 proteins, mitogen-activated protein kinase kinase (Mek) and some other proteins (8–11, 44–46). Thus far, we did not find any polymorphism in these domains of interactions, suggesting that all Raf proteins interact with the same cellular proteins. Accordingly, we reported that different B-Raf isoforms associate with Mek-1 and phosphorylate this protein at the two specific activating serine residues 218 and 222 (25). Recent results suggest that different B-Raf isoforms interact with activated p21^{ras} (23). B-Raf proteins appear to constitute the major mitogen-activated protein kinase kinase kinase (Mek kinase) activity in PC12 cells (21, 22) and to be involved in activation of this kinase cascade by nerve growth factor (21). Conversely, Mek-1 activation in the brain appears to depend on the presence and association of B-Raf proteins with activated p21^{ras} (23, 24). Taken together, these results indicate that the different B-Raf proteins act as signal transducers between Ras-GTP and Mek.

Thus, the variable region (between CR2 and CR3), which displays low similarity among members of the Raf family, could direct interactions of B-Raf isoforms with specific effectors. There have been only few reports showing that the presence of an alternatively spliced exon could modify the properties of a kinase. The presence of a neurospecific exon in the c-Src protein increases its specific kinase activity (47). In the tyrosine kinase receptor TrkC, insertion of alternatively spliced exons results in the loss of its ability to phosphorylate phospholipase

C γ and phosphatidylinositol 3-kinase (48). In calmodulin kinase, an alternative splicing introduces a nuclear localization signal that targets a calmodulin kinase isoform to the nucleus (43). Therefore, it is possible that the presence of alternatively spliced exons could modulate the B-Raf kinase activity, the specificity of its substrates, or its targeting within the cell.

It is not clear whether the B-Raf proteins are implicated in differentiating or proliferating signal transduction pathways. It is interesting that, in testes, B-raf transcripts are detected only in pachytene spermatocytes and more abundantly in post-meiotic spermatids as shown by *in situ* hybridization (26). We also detected B-Raf proteins more abundantly in neural tissues, where the vast majority of cells are postmitotic. These results suggest that B-Raf proteins could be involved in differentiation rather than in proliferation processes.

Acknowledgments—We thank F. Lamballe and P. Vernier for critical reading of the manuscript, A. Rotig for providing the child brain biopsy, and J. C. Cavadore for helpful advice on antibody strategies. We thank F. Arnouilh for help with the preparation of the manuscript and J. P. Bouillot and O. Champion for assistance in preparation of figures.

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