Members of the Raf oncogene family encode serine/threonine protein kinases, which activate the mitogen-activated protein kinase kinase MEKs (MAPK or ERK kinases) through direct interaction and phosphorylation. Several recent studies have revealed interesting differences between two members of this family, Raf-1 and B-Raf, regarding their activation, regulation, and kinase activity. In particular, B-Raf was shown to display higher MEK kinase activity than Raf-1. By using both two-hybrid analysis and coimmunoprecipitation experiments, we demonstrate here that B-Raf also markedly differs from Raf-1 by a higher affinity for MEK. We previously reported that the B-raf gene encodes multiple protein isoforms resulting from complex alternative splicing of two exons (exons 8b and 10) located upstream of B-Raf kinase domain. In the present study, we show that these naturally occurring modifications within the protein sequence markedly modulate both the biochemical and oncogenic properties of B-Raf. The presence of exon 10 sequences enhances the affinity for MEK, the basal kinase activity, as well as the mitogenic and transforming properties of full-length B-Raf, whereas the presence of exon 8b sequences seems to have opposite effects. Therefore, alternative splicing represents a novel regulatory mechanism for a protein of the Raf family.

The mitogen-activated protein (MAP) kinase cascades are membrane-to-nucleus signaling modules highly conserved from yeast to vertebrates and involved in multiple physiological processes (1, 2). Among the different MAP kinase cascades thus far identified in eucaryotes, one has been found only in metazoans, and its activation by extracellular mitogenic or differentiating signals is mediated through Ras GTPases. In this cascade, proteins of the Raf family function as MAP kinase kinase kinases (MAPKKKs). Raf proteins have been shown to phosphorylate and activate MAP kinase kinases (MAPKKs) called MEKs (MAPK or ERK kinases), which in turn phosphorylate and activate MAP kinases (MAPKs) called extracellular signal-regulated kinases (ERKs). In vertebrates, three Ras (H-Ras, N-Ras, and K-Ras) (3), two ERKs (ERK-1 and ERK-2) (4), two MEKs (MEK-1 and MEK-2) (5–7), and three Raf proteins (A-Raf, B-Raf/Rmil, and Raf-1/Mil) (8–10) have been so far characterized. Moreover, Kar, a novel protein kinase related to Raf proteins, was recently shown to regulate this pathway through its interaction with different kinases of the module (11–13).

An additional level of diversity is provided by tissue or cell-type distribution of each of these kinases. In this regard, B-Raf proteins are probably the most tightly regulated among these kinases. The B-raf gene encodes at least ten protein isoforms resulting from complex alternative splicing and displaying tissue-specific expression (14, 15). The highest level of expression was found in neural tissues and one subclass of B-Raf isoforms, which contain amino acid sequences encoded by the 120-base pair exon 10 (10), is specifically expressed in these tissues (14). Therefore, the question of the specificity of each B-Raf isoform in signaling pathways remained to be addressed.

This increase in diversity of the Ras/ERK pathway components might allow an increase in the different possibilities of the cascade regulation as well as an involvement in distinct signaling pathways. In this regard, targeted disruption of murine A-raf and B-raf resulted in distinct phenotypes, suggesting a limited redundancy in the functions of Raf proteins during development (16, 17). In addition, several lines of evidence indicate that Raf-1 and B-Raf proteins are differentially regulated and differ in their ability to activate the MAP kinase pathway in different cell systems. Although both proteins can form complexes with Ras-GTP (18–21), they can undergo differential activation at the plasma membrane. Thus, it was recently reported that, in COS cells, B-Raf is maximally activated by oncogenic Ras alone, whereas Raf-1 requires both Ras activation and tyrosine phosphorylation (22). In PC12 cells, activation of another GTPase, Rap-1, by mutation or by the cAMP-dependent protein kinase A, resulted in the selective activation of B-Raf and subsequent stimulation of the MAPK/ERK cascade in a Ras-independent manner, whereas Raf-1 was inhibited under the same conditions (23).

Raf-1 and B-Raf proteins not only differ in their regulation by upstream activators but also in their ability to activate downstream effectors. Thus, B-Raf proteins seem to be the major MEK activators in neural cells (18, 21, 24, 25) and possibly also in fibroblasts and hematopoietic cell types (15, 20), although both B-Raf and Raf-1 are expressed and activated in these cells. The differences between these two proteins in their intrinsic abilities to activate MEK in vivo could be explained, at least in part, by their differential ability to phosphorylate MEK in vitro (22, 26). In addition, our recent studies on the interactions of B-Raf with different signaling proteins in the yeast two-hybrid system suggested that although B-Raf...
B-Raf Regulation by Alternative Splicing

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions**—The two-hybrid plasmids containing the complete coding sequence of B1-, B2-, B3-, and B4-Raf isoforms, Raf-1, MEK-1, and MEK-2 proteins were previously described (19). The pSVL-derived constructs expressing the different B-Raf isoforms were described elsewhere (27). The pSVL/Raf-1 construct was obtained by subcloning the XhoI-BamHI fragment of human Raf-1 from pBluescript/Raf-1 into the XhoI-BamHI sites of pSVL (Amersham Pharmacia Biotech). Plasmids expressing the HA1 epitope-tagged Raf-1 and B-Raf proteins were obtained as follows. The EcoRI B-Raf fragment from pGBl-9B1-Raf (19) and the EcoRI-XhoI Raf-1 fragment from pBluescript/Raf-1 were cloned into the EcoRI and EcoRI-XhoI sites, respectively, of a pcDNA3-derived construct containing the HA1-epitope sequence followed by an EcoRI site allowing an in-frame fusion with Raf proteins (kindly provided by Dr. Jean de Gunzburg).

Recombinant baculoviruses expressing the different Raf proteins were obtained as follows. The EcoRI fragments containing the complete coding sequence of the different B-Raf isoforms and the EcoRI-PstI fragment containing the complete coding sequence of the human Raf-1 protein, obtained from pGB79B1-, B2-, B3-, B4-Raf, and pGB79Raf-1, respectively (19), were inserted into the pcAHIT/A vector (Baculo-gold kit from Pharmingen).

The HindIII fragments derived from pBluescript/B1-, B2-, and B4-Raf and the HindIII-XhoI fragment derived from pBluescript/Raf-1 were inserted into the pCR/Blu vector (Invitrogen) to generate pCR/Raf/ B1-, B2-, B3-, B4-Raf and Raf-1, respectively.

The Ras CAAX sequence was added to the COOH-terminal extremity of B1-Raf and B3-Raf by polymerase chain reaction amplification as described previously (28), using the sequence encoding the 19 carboxy-terminal amino acids of human K-Ras present in the pZnCK plasmid (kindly provided by Dr. Jean de Gunzburg) as a template. The SpH1-XhoI fragment of pEF/Raf-CAAX (kindly provided by Dr. Julian Dowward), encoding the COOH-terminal end of human Raf-1 fused to the Ras CAAX sequence, was cloned in place of the corresponding fragment of the normal human Raf-1 cloned into the pBluescript/Raf-1. B1-Raf-CAAX, B3-Raf-CAAX, and Raf-1-CAAX cDNAs were subsequently cloned into the pRcRSp vector.

**RESULTS**

The Ras CAAX sequence is added to the COOH-terminus of B1-Raf and B3-Raf by polymerase chain reaction amplification as described previously (28), using the sequence encoding the 19 carboxy-terminal amino acids of human K-Ras present in the pZnCK plasmid (kindly provided by Dr. Jean de Gunzburg) as a template. The SpH1-XhoI fragment of pEF/Raf-CAAX (kindly provided by Dr. Julian Dowward), encoding the COOH-terminal end of human Raf-1 fused to the Ras CAAX sequence, was cloned in place of the corresponding fragment of the normal human Raf-1 cloned into the pBluescript/Raf-1. B1-Raf-CAAX, B3-Raf-CAAX, and Raf-1-CAAX cDNAs were subsequently cloned into the pRcRSp vector.

**Fig. 1.** Comparison of B-Raf and Raf-1 interaction with MEK and basal kinase activities. A, quantitative analysis of the interaction of Raf-1 and B-Raf with MEK-1 and MEK-2 in the yeast two-hybrid system. Raf and MEK proteins were cloned in frame with Gal-4 activation and DNA binding domains, respectively, and the constructs were used to transform the yeast Y187 strain. Yeast cotransformants were subjected to an in vitro kinase assay using GST-MEK-1 as a substrate, and Raf-1 interacted similarly with activated Ras, B-Raf protein isoforms display a stronger MEK-binding capacity than that of Raf-1 (19). Thus, differences in both kinase activity and affinity of Raf proteins toward MEK might explain that B-Raf is a much stronger activator of MEK in vivo. This is supported by the profound effect of B-Raf null mutation resulting in embryonic lethality between 10.5 and 12.5 days after fertilization (17).

To address this question more thoroughly, we used HA1 epitope-tagged B-Raf and Raf-1 to compare both their basal MEK kinase activity and affinity for MEK. We found that B-Raf not only displays a higher basal kinase activity than Raf-1 in different cell backgrounds but also binds MEK with a stronger affinity than Raf-1 by using both a quantitative assay in the two-hybrid system and coimmunoprecipitation experiments.

Because the B-Raf amino-terminal region is subjected to alternative splicing of two exons (exon 8b and exon 10), resulting in the synthesis of four distinct isoforms (14), we also investigated whether the presence of these alternative sequences could influence the biochemical and biological properties of B-Raf. We show that alternative splicing modulates the basal MEK kinase activity as well as the oncopgenic properties of full-length B-Raf protein isoforms. Specifically, we found that the exon 10-containing B3 isoform displays higher affinity for MEK, higher in vitro MEK kinase activity, and increased oncopgenic properties. On the other hand, the presence of exon 8b sequences seems to have opposite effects on B-Raf activity. Our results provide evidence that alternative splicing constitutes a novel mechanism for the regulation of B-Raf activity.
The EcoRI fragments derived from pGBT9/B1-, B2-, B3-, B4-Raf and the EcoRI-SalI fragment derived from pGBT9/Raf-1 were inserted into the pBabe-puro retrovirus vector (29) to generate pBabe/B1-, B2-, B3-, B4-Raf and pBabe/Raf-1, respectively. The EcoRI fragment from pCDNA/RasV12 (kindly provided by Dr. Julian Downward), containing the coding sequence of an activated human H-Ras, was inserted into the pBabe-puro to generate pBabe/RasV12. The pBabe/v-Rmil construct, encoding a recombinantly activated kinase domain of the avian homolog of B-Raf, was obtained as follows. The SacI fragment derived from the R5Rmil cDNA (30) was blunt-ended with the T4 DNA polymerase and inserted into the SnaB1 site of the pBabe-puro vector.

Quantitative β-Galactosidase Assay in Yeast Two-hybrid System—

The Y187 strain was cotransformed with the different pGBT9- and pGADGH (or pGADMOD)-derived constructs, using the lithium acetate method as described previously (27). The reported values represent means of six independent transformants (means ± standard deviation). C, communoprecipitation analyses of MEK-1 and B-Raf isoforms in COS-1 cells. COS-1 cells were transfected with pSVL-derived constructs encoding the different B-Raf isoforms as indicated, and protein expression was analyzed 48 h later. Cell lysates were normalized for the expression of B-Raf proteins and then immunoprecipitated with either the IS11 anti-B-Raf polyclonal antibody or an anti-MEK-1 polyclonal antibody as indicated (IP), and immune complexes were resolved on SDS-PAGE for Western blotting analysis as indicated (WB). Results are representative of at least three independent experiments.

In Vitro Protein Kinase Assays—Immune complexes prepared from COS-1 and Sf9 cells, as described above, were washed once with kinase buffer (20 mM Hepes, pH 7.2, 10 mM MgCl2, 10 mM MnCl2) and then incubated in kinase buffer supplemented with 10 μCi of [γ-32P]ATP (3000 Ci/mmol, Amersham) and 1–2 μg of purified GST-MEK-1 (Upstate Biotechnology Inc.). After incubation for 30 min at 30 °C, reactions were stopped by addition of Laemmli sample buffer, resolved by SDS-PAGE, and transferred to Immobilon-P membranes (Millipore Corp.). Membranes were first submitted to autoradiography before proceeding to Western blotting analysis. Membranes were probed with either the 12CA5 anti-HA1 monoclonal antibody (Boehringer Mannheim), or 6 μl of an anti-MEK-1 monoclonal antibody (a generous gift of Dr. Jacques Pouysségur), and coupled to 50 μl of pansorbin (Calbiochem) for 2 h. Immunoprecipitates were washed under stringent conditions as described previously (27). For communoprecipitation analyses, immune complexes were resolved on SDS-PAGE, transferred to Immobilon-P membranes (Millipore Corp.), and probed with the anti-B-Raf, the anti-β-HA-epitope, or the anti-MEK-1 antibodies at a 1:2000 dilution and visualized by chemiluminescence as recommended by the manufacturer (Amersham).

Recombinant baculovirus stocks expressing the different Raf proteins were produced by transfecting the pACHTLA-derived constructs into Sf9 cells maintained in TC100 medium containing 10% fetal calf serum using the Baculo-Gold kit (Pharmingen). For protein production, 3 × 106 cells were infected with the different recombinant baculoviruses at a multiplicity of infection of 10. Cells were lysed 40 h after infection in insect cell lysis buffer (containing 10 mM Tris, pH 7.5, 130 mM NaCl, 1% Triton X-100, 10 mM NaF, 10 mM NaPi, 10 mM NaPPi). Cell lysates were incubated with the anti-6×His monoclonal antibody (CLON-TECH) and coupled to 50 μl of pansorbin (Calbiochem) for 2 h. Immune complexes were washed under stringent conditions as described previously (27).
Basal Kinase Activities—We previously reported that B-Raf and Raf-1 displayed comparable Ras-binding capacity, using a β-galactosidase filter assay in the yeast two-hybrid system. In contrast, we observed that these two proteins markedly differed by the strength of their interaction with MEK proteins, suggesting that B-Raf affinity for MEK-1 and MEK-2 was higher than that of Raf-1 (19). To measure this difference, we used a quantitative liquid β-galactosidase assay in the two-hybrid system, a method previously shown to provide a good correlation with the affinity of protein interactions measured in vitro (35). The comparison was done between Raf-1 and B1-Raf, an isoform of B-Raf devoid of alternatively spliced exons 8b and 10. Results presented in Fig. 1A show that the β-galactosidase activity resulting from B1-Raf/MEK interaction was 10 times higher than that of Raf-1/MEK interaction, the two proteins being expressed at similar levels in yeast transformants as verified by Western blotting analysis (data not shown). To confirm this observation by another experimental approach, we performed coimmunoprecipitation experiments using epitope-tagged Raf proteins ectopically expressed in COS-1 cells (Fig. 1B). The HA1-B1-Raf protein was easily detected in immune complexes immunoprecipitated with an anti-MEK-1 antibody directed against the COOH-terminal extremity of Raf-1 (Santa Cruz) at a 1:2000 dilution. A

**FIG. 3.** Alternative splicing modulates B-Raf basal kinase activity. **A**, in vitro basal kinase activity of baculovirus-expressed full-length Raf-1 and B-Raf isoforms. SF9 insect cells were infected with baculoviruses expressing His-tagged Raf-1 or B-Raf isoforms depicted in Fig. 2A, and cell lysates were immunoprecipitated with an anti-6×His monoclonal antibody. Immunoprecipitates were subjected to in vitro kinase assay using inactive GST-MEK-1 as a substrate for 30 min, separated on SDS-PAGE, and transferred to immobilon-P membranes. MEK phosphorylation as well as Raf protein autophosphorylation (Auto-P) levels were revealed by autoradiography (kinase). Membranes were then submitted to Western blotting analysis (WB) using the anti-6×His monoclonal antibody. **B**, time-course experiment. In vitro kinase assays were performed as described in A for each isoform, and samples of the reactions were stopped at different times of incubation as indicated. GST-MEK phosphorylation levels were quantified using a PhosphorImager (Molecular Dynamics).

**RESULTS**

Comparison of B-Raf and Raf-1 Interaction with MEK and Basal Kinase Activities—We previously reported that B-Raf and Raf-1 displayed comparable Ras-binding capacity, using a β-galactosidase filter assay in the yeast two-hybrid system. In contrast, we observed that these two proteins markedly differed by the strength of their interaction with MEK proteins, suggesting that B-Raf affinity for MEK-1 and MEK-2 was higher than that of Raf-1 (19). To measure this difference, we used a quantitative liquid β-galactosidase assay in the two-hybrid system, a method previously shown to provide a good correlation with the affinity of protein interactions measured in vitro (35). The comparison was done between Raf-1 and B1-Raf, an isoform of B-Raf devoid of alternatively spliced exons 8b and 10. Results presented in Fig. 1A show that the β-galactosidase activity resulting from B1-Raf/MEK interaction was 10 times higher than that of Raf-1/MEK interaction, the two proteins being expressed at similar levels in yeast transformants as verified by Western blotting analysis (data not shown). To confirm this observation by another experimental approach, we performed coimmunoprecipitation experiments using epitope-tagged Raf proteins ectopically expressed in COS-1 cells (Fig. 1B). The HA1-B1-Raf protein was easily detected in immune complexes immunoprecipitated with an anti-MEK-1 antibody, confirming the strong binding capacity of B-Raf with endogenous MEK. In contrast, the presence of HA1-Raf-1 protein in MEK-1 immunoprecipitates was not detected in the same conditions.

We also examined the basal kinase activity of HA1-tagged
Raf proteins expressed in COS-1 cells, using GST-MEK as a substrate. Results presented in Fig. 1 show that HA1-B-Raf displays a high basal kinase activity toward MEK-1, whereas that of Raf-1 is about 10-fold lower, in agreement with the results of Marais et al. (22). This observation is not specific of the cell type used, as similar results were obtained with baculovirus-expressed Raf proteins in Sf9 insect cells (Fig. 3).

Alternative Splicing Modulates Both B-Raf Interaction with MEK and B-Raf Basal Kinase Activity—Because our results indicated that B-Raf markedly differs from Raf-1 by its ability to interact with and phosphorylate MEK, we next investigated whether alternative splicing could modulate the biochemical properties of B-Raf isoforms. We previously showed that the region upstream of the B-Raf kinase domain is subjected to complex alternative splicing (10, 14). Specifically, we identified two alternatively spliced exons, designated 8b and 10, encoding 12 and 40 amino acids respectively, located within the region between the CR2 and CR3 domains of B-Raf proteins (Fig. 2A), and we showed the existence of protein isoforms containing either or both exons (14). To investigate the influence of these alternative sequences on B-Raf protein activities, we used cDNAs encoding each of the B1-, B2-, B3-, and B4-Raf isoforms (Fig. 2A) to compare their MEK-binding capacity and levels of kinase activity.

We cloned the full-length B-Raf isoforms in frame with the Gal-4 activation domain and assayed their interaction with MEK-1 and MEK-2, cloned in frame with the Gal-4 DNA binding domain, in the yeast two-hybrid system (19). We found that the β-galactosidase activity produced by B3- and B4-Raf, both containing exon 10, was about three times higher than that obtained with B1- and B2-Raf isoforms, which do not contain this exon (Fig. 2B), although all isoforms were expressed at similar levels in yeast transformants (data not shown). To confirm this by another experimental approach, we compared the ability of B-Raf isoforms to form complexes with endogenous MEK-1 in COS-1 cells by coimmunoprecipitation experiments. All B-Raf isoforms were readily detected in immune complexes immunoprecipitated with an anti-MEK-1 antibody (Fig. 2C). However, B3- and B4-Raf, which contain exon 10, reproducibly coimmunoprecipitated with MEK with higher efficiency than that of B1- and B2-Raf isoforms, although the differences were not as marked as those observed in the two-hybrid system. Taken together, these results indicate that the presence of exon 10 increases the efficiency of B-Raf/MEK interaction, whereas that of exon 8b alone does not seem to influence the strength of this interaction.

We also studied the influence of exon 8b and exon 10 on the basal kinase activity of B-Raf isoforms by using proteins produced in insect cells infected with recombinant baculoviruses. B-Raf proteins were immunoprecipitated from cell lysates prepared 40 h after infection with a monoclonal anti-histidine tag antibody. We then measured in vitro kinase activity of immune complexes by using an enzymatically inactive recombinant GST-MEK-1 as a substrate (Fig. 3). The highest in vitro basal MEK kinase activity was found for the B3-Raf isoform. In addition, B3- and B4-Raf displayed higher basal kinase activity in comparison with their respective counterparts B1- and B2-Raf lacking exon 10. On the other hand, the kinase activity of B2- and B4-Raf was lower than that of their respective counterparts B1- and B3-Raf, lacking exon 8b. Taken together, these results suggest that the presence of sequences encoded by exon 10 results in an increase of B-Raf basal kinase activity toward MEK in vitro, whereas those encoded by exon 8b seem to have an opposite effect.

Exon 10 Sequences Confer Increased Mitogenic and Transforming Properties to Full-length B-Raf Proteins—To investi-
gate whether alternative splicing could also modulate the oncogenic properties of B-Raf, we compared the ability of full-length B-Raf isoforms to induce sustained proliferation of differentiating chicken embryonic NR cells and to transform mouse NIH3T3 fibroblasts.

We previously reported that post-mitotic avian NR cells acquire sustained proliferative capacity following infection with retroviruses encoding amino-terminally truncated Raf-1/Mil and B-Raf/Rmil proteins (36–38). We also showed that v-Src mutants displaying reduced transforming properties retain full capacity to induce NR cell proliferation (39). Therefore, these cells represent sensitive indicators for the detection of mitogenic properties, even in the absence of gross morphological alterations (11).

cDNAs encoding each of the B-Raf isoforms and Raf-1 were cloned into the pRcRSV expression vector and used to transfect NR cultures dissected from 8-day chick embryos. Occurrence of foci of proliferating NR cells was examined 20 days after transfection and G418 selection (Fig. 4). In NR cultures transfected with an empty vector, G418 resistant cells remained isolated and did not give rise to foci since they lacked proliferative capacity. In contrast, multiple foci of dividing cells were visible in cultures transfected with cDNAs encoding full-length B-Raf and Raf-1 proteins. The number and size of foci were significantly higher in cultures transfected with plasmids encoding B3- and B4-Raf isoforms containing exon 10, as compared with those transfected with plasmids encoding the B1-, B2-Raf, or Raf-1 (Fig. 4).

Our previous results indicated that retroviruses encoding Raf-1/Mil or B-Raf/Rmil activated by amino-terminal truncation displayed comparable mitogenic properties in NR cells. We examined whether constitutive activation of full-length B-Raf and Raf proteins would abolish the differences in their basal mitogenic capacities. Therefore, we constructed expression vectors encoding each of the B1-Raf, B3-Raf, and Raf-1 proteins fused to the CAAX sequence of K-Ras, previously shown to constitutively activate the oncogenic properties of full-length Raf-1 (28, 40). We found that transfection of NR cells with cDNA encoding any of the Raf-CAAX proteins resulted in marked and comparable increase in the number of proliferating colonies (Fig. 4).

We next investigated whether proliferation of NR cells transfected with constructs encoding full-length Raf proteins could be sustained through several passages, as observed with activated oncogenes. Therefore, we compared the cumulative growth capacity of G418-resistant cells in each transfected culture during four passages (Fig. 5). We found that the number of NR cells transfected with plasmids encoding Raf-1, B1-, or B2-Raf proteins decreased with passages. In contrast, NR cells expressing the B3- or B4-Raf isoforms were able to sustain active division, as shown by the steady increase in cell number with passages. Interestingly, the cumulative number of cells during the third and fourth passages was significantly higher in B3-Raf transfected cultures, as compared with cells transfected with B4-Raf expressing vector. This suggests that amino acids encoded by exon 8b are able to antagonize, to a certain extent, the potent mitogenic effect correlated with the presence of exon 10, when expressed alone. Under these conditions, cultures expressing Raf-1-CAAX or B1-Raf-CAAX proteins maintained a high level of proliferation and, in addition, were morphologically transformed (data not shown). Therefore, fusion with CAAX sequence confers upon Raf-1 and B1-Raf proteins the ability to induce long-term NR cell proliferation.

We also tested the effects of Raf protein overexpression on mouse fibroblast transformation. Therefore, we cloned cDNAs encoding full-length Raf-1 and B-Raf proteins, as well as activated Ha-Ras and v-Rmil, an amino-terminally truncated version of the avian homolog of B-Raf (30), into the pBabe-puro retroviral expression vector. Plasmid DNAs were transfected into the packaging Bosc 23 cells (34) to generate replication-defective retroviruses used to infect NIH3T3 cells. Control cultures were infected with retroviruses containing only the selection marker. Puromycin-resistant cells were then assayed for their ability to form colonies in soft agar (Fig. 6B). We found that cells overexpressing B3- and B4-Raf isoforms formed numerous and large colonies, as compared with control cells and with cells expressing comparable levels of Raf-1, B1-, and B2-Raf, which gave rise to smaller and less abundant colonies (Fig. 6, B and C). Again, we observed that cells overexpressing exon 8b-containing B2- and B4-Raf proteins formed less colonies than those expressing B1- and B3-Raf, respectively (Fig. 6C). Therefore, we provide here the first evidence that full-length Raf proteins are able to induce anchorage-independent growth when overexpressed in NIH3T3 cells. However, the number
and size of colonies obtained with these proteins were significantly smaller than those observed in cultures infected with activated Ras or v-Rmil expressing retroviruses.

**DISCUSSION**

Several recent studies have revealed interesting differences between Raf-1 and B-Raf regarding their activation, regulation, and kinase activity. In particular, B-Raf was shown to display higher MEK kinase activity than Raf-1 (19, 22, 26). Our previous study, using two-hybrid filter assays, suggested that B-Raf affinity toward MEK was higher than that of Raf-1, whereas both proteins were found to bind Ras with similar affinities (19). In the present work, we confirmed these observations by two different experimental approaches. By using a quantitative liquid β-galactosidase assay in the two-hybrid system, we showed that the β-galactosidase activity resulting from B1-Raf/MEK interaction is 10 times higher than that of Raf-1/MEK interaction. Accordingly, the two proteins markedly differ by their ability to form complexes with endogenous MEK-1 in COS-1 cells, as shown by coimmunoprecipitation of epitope-tagged proteins. Consistently, no evidence for the existence of Raf-1/MEK complexes by coimmunoprecipitation experiments has been reported thus far.

In agreement with the results of Marais et al. (22), we also confirmed that the two proteins differ by their basal kinase activity. Thus, baculovirus B-Raf expressed in Sf9 cells as well as HA1-tagged B-Raf expressed in COS-1 cells display higher basal kinase activity toward MEK-1 in comparison with that of Raf-1 under the same conditions. Further analyses are required to identify amino acid sequences that are specifically responsible for these differences. Raf-1 was previously shown to be activated by Src family kinases through the phosphorylation of two tyrosine residues (Tyr^{447} and Tyr^{448}) at the corresponding positions, substitution of these residues by tyrosine does not significantly reduce B-Raf catalytic activity (22, 43). We also compared B-Raf/ Asp^{447}Asp^{448} and B-Raf/Thr^{447}Thr^{448}, and we found that neither the affinity nor the basal kinase activity of B-Raf was decreased by these mutations under experimental conditions used in this study (data not shown).

As the region located upstream of the B-Raf kinase domain is subjected to alternative splicing of two exons (exon 8b and exon 10), resulting in the synthesis of four distinct isoforms (14), we postulated that the presence of these alternative sequences could influence the biochemical and biological properties of B-Raf. The results presented in this study demonstrate that these naturally occurring modifications, within the protein sequence, modulate their affinity for MEK and their catalytic activity, as well as their oncogenic properties.

Thus, we show that the presence of sequences encoded by the alternatively spliced exon 10 increases both B-Raf affinity and basal kinase activity toward MEK. In contrast, the alternatively spliced exon 8b seems to decrease B-Raf basal kinase activity but has no significant effect on its affinity for MEK. These two exons are located at the junction between the regulatory and the catalytic domains of the protein. It was proposed that, in the inactive conformation of Raf-1, the amino-terminal region is folded in contact with the catalytic domain (44). In this model, the “linker” sequence between the conserved domains could function as a hinge in the transition between the closed inactive and open active conformations (Fig. 7). This function is consistent with the presence of several regulatory phosphorylation sites in this region of Raf proteins (41, 42, 45). A similar regulation by conformational change has been described for the Src protein. In this case, the linker domain, between the SH2 and kinase domains, contributes to the stability of the closed conformation by maintaining the SH3 and
kinase domains of the protein in tight contact (46). The primary sequences of the CR1, CR2, and CR3 domains are well conserved among the Raf family. In contrast, the linker region is specific of each Raf protein and does not display homology with known proteins. It is, therefore, conceivable that sequence variation in this region could influence transition between the inactive and active states (Fig. 7). The presence or absence of alternative exons in B-Raf may add to the variability of this region, which contributes to regulation of the kinase. Thus, exon 10 sequences may play a role in facilitating and stabilizing the active conformation of the kinase, whereas exon 8b peptidic sequences may have the opposite effect (Fig. 7). An alternative explanation for the observed differences would be that exons 8b and 10 sequences are involved in other interactions with specific signaling molecules or determine different intracellular protein localization. It will be of interest to address the latter issue because, although all B-Raf isoforms contain the CR1 domain involved in the binding to the effector domain of Ras family members, a particular subcellular localization could specify a particular B-Raf isoform to a specific small GTPase. For example, Ras and Rap1 proteins can be localized in different cell compartments depending on the cell type (47).

We have also shown that the presence of these alternatively spliced sequences modulate the biological properties of B-Raf proteins. Our results provide the first evidence that overexpression of full-length Raf family proteins is sufficient to induce sustained proliferation of quiescent NR cells and transformation of NIH3T3 fibroblasts in the absence of mutations or amino-terminal deletion. Our data appear to establish a rough correlation between the levels of affinity and in vitro kinase activity toward MEK on the one hand and the extent of oncogenic properties on the other. Thus, cells overexpressing the exon 10-containing B3- and B4-Raf display significantly higher oncogenic potential. Conversely, the presence of exon 8b, which decreases B-Raf kinase activity, but not its affinity toward MEK, also seems to decrease, to a limited extent, the oncogenic properties of B2- and B4-Raf isoforms. However, such a strict correlation between the levels of in vitro kinase activity and the extent of transformation was not always verified, as B2-Raf and Raf-1 markedly differ in their basal kinase activity but induce comparable mitogenic and transforming properties. This strongly suggests that Raf-1 is subjected to additional regulations in vivo to reach an activation threshold similar to that of B-Raf.

This study provides the first evidence that, in addition to post-translational modifications such as phosphorylation/dephosphorylation, alternative splicing represents a novel mechanism regulating the activity of a Raf family protein. A similar mode of regulation was described for the c-Src tyrosine kinase. Thus, the presence of a neural specific, alternatively spliced exon in the Src protein was shown to increase both the kinase activity and transforming properties of this protein (48). We have shown that exon 10-containing proteins are almost exclusively detected in neural tissues (14). The amino acid sequence encoded by this exon is perfectly conserved in chicken, quail, mouse, and human species (10, 14). It would be, therefore, interesting to investigate whether the higher activity of exon 10-containing B-Raf isoforms accounts for specific function(s) in these tissues.

Raf family members share common events involved in their activation, such as interaction with Ras. However, this report reinforces the notion that unique regulatory events are also involved in the activation of each of the Raf kinases. Thus, Raf-1 can be further activated upon phosphorylation by tyrosine kinases of the Src family, which is not required for B-Raf activation (22). Such a multistep activation may impose a tighter control on the regulation of Raf-1, the basal kinase activity of which is low. In contrast, B-Raf, like ancestor D-raf and lin45 genes (in Drosophila and Caenorhabditis elegans, respectively), is not submitted to the same regulations. Therefore, alternative splicing may well be an additional and important regulatory mechanism to control B-Raf activity.

Acknowledgments—We thank Dr. Jacques Ghysdael for help in baculovirus preparation, Anthony Boureux for helpful advice on transformation assays in NIH3T3 cells, and Dr. Sylvie Nessler for helpful discussions. We also thank Drs. Jean de Gunzburg and Julian Downward for providing reagents used in this study.

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