Tissue and Species Distribution of mRNA Encoding Two ADP-ribosylation Factors, 20-kDa Guanine Nucleotide Binding Proteins†

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ABSTRACT: Cholera toxin catalyzed ADP-ribosylation of $G_{s\alpha}$, the stimulatory guanine nucleotide binding protein of the adenylyl cyclase system, is enhanced by ~20-kDa guanine nucleotide binding proteins, termed ADP-ribosylation factors or ARFs. ARF is an allosteric activator of the A1 catalytic protein of the toxin. Bovine ARF cDNA clones, ARF-1 isolated from adrenal (Sewell & Kahn, 1988) and ARF-2B from retina (Price et al., 1988), exhibit nucleotide and deduced amino acid sequences that are 80% and 96% identical, respectively, in the coding region. To determine tissue and species distribution of ARF-like mRNAs, bovine ARF-2B and human ARF-1 cDNAs and 30- or 48-base oligonucleotide probes that distinguish between ARF-1 and ARF-2B cDNAs in coding and 3′-untranslated regions were used for Northern analysis of poly(A+) RNA from different tissues and species. On the basis of hybridization with specific oligonucleotide probes, all bovine tissues contained mRNAs of 1.7 and 2.1 kb that were related to ARF-1 and ARF-2B, respectively. Northern analysis of brain poly(A+) RNA from different species with ARF-2B and ARF-1 cDNAs at low stringency demonstrated several bands varying in size from 0.9 to 3.7 kb. A 1.7-kb band consistently hybridized with an ARF-1 30-base coding-region probe but not with a probe for the 3′-untranslated region. Similar ARF-2B oligonucleotide probes did not hybridize with rat, mouse, rabbit, or human brain mRNA. Cleavage of ARF-2B cDNA with *Pvu*II generated two fragments, one containing coding and the other 3′-noncoding region. Only the coding-region cDNA hybridized with brain poly(A+) RNA from species other than bovine, consistent with other evidence that the ARF-2B coding region is more highly conserved than the 3′-noncoding region. On the basis of these data and the differences in nucleotide sequences of ARF-1 and ARF-2B cDNAs, there appear to be at least two highly homologous ARF genes, expressed in a variety of species and tissues.

Cholera toxin, a secretory product of *Vibrio cholera*, is responsible for the devastating diarrheal syndrome characteristic of cholera (Finkelstein, 1973; Carpenter, 1980; Kelly, 1986). The enterotoxin causes abnormalities in fluid and electrolyte flux by catalyzing the ADP-ribosylation in intestinal cells of $G_{s\alpha}$, a guanine nucleotide binding protein that activates the adenylyl cyclase catalytic unit and may regulate ion flux as well (Birnbaumer, 1987; Casey & Gilman, 1988; Moss & Vaughan, 1988). Effects of cholera toxin on the adenylyl cyclase system are enhanced by membrane and soluble factors (Enomoto & Gill, 1980; Le Vine & Cuatrecasas, 1981; Enomoto & Asakawa, 1982; Pinkett & Anderson, 1982; Schleifer et al., 1982; Gill & Meren, 1983; Kahn & Gilman, 1984, 1986; Tsai et al., 1987, 1988a). One of these, initially purified from liver and subsequently from bovine brain membranes, was a 21-kDa guanine nucleotide binding protein that enhanced cholera toxin catalyzed ADP-ribosylation of $G_{s\alpha}$ and was termed ADP-ribosylation factor or ARF (Kahn & Gilman, 1984, 1986).

Two soluble ARF-like proteins have been purified from bovine brain (Tsai et al., 1988a); these are similar in size, guanine nucleotide binding properties, and immunoreactivity to the membrane species (Tsai et al., 1987, 1988a,b; Kahn et al., 1988; Bobak et al., 1989a). It was shown that both membrane and soluble ARFs, in addition to enhancing cholera toxin catalyzed ADP-ribosylation of $G_{s\alpha}$, also enhanced other toxin-catalyzed reactions such as the ADP-ribosylation of simple guanidino compounds or proteins unrelated to the cyclase system and auto-ADP-ribosylation of the toxin A1 protein (Tsai et al., 1987, 1988a). Kinetic analysis revealed that ARF enhanced the NAD:agmatine ADP-ribosyltransferase activity of the toxin by increasing affinity for both substrates with little effect on V_{max} (Noda et al., 1989). It was concluded that ARF activation of the toxin is, in fact, independent of $G_{s\alpha}$ and results from direct effects on the allosteric properties of the toxin catalytic unit.

ARF cDNA clones have been isolated from bovine retinal (ARF-2B) and adrenal (ARF-1) and human cerebellum (ARF-1) libraries (Price et al., 1988; Sewell & Kahn, 1988; Bobak et al., 1989b). Although the deduced amino acid sequences derived from the ARF-2B and ARF-1 cDNAs are very similar, the nucleotide sequences of the two types of clones differ considerably throughout the coding region, and they are apparently the products of two different genes. To determine the size(s) and number of ARF-2B- and ARF-1-like mRNAs as well as their relatedness, poly(A+) RNA from several tissues and species was analyzed by hybridization with specific oligonucleotide and cDNA probes.

Experimental Procedures

Materials. Random primed DNA labeling kits and *Pvu*II were purchased from Boehringer Mannheim; terminal deoxynucleotidyl transferase and RNA standards were from Bethesda Research Laboratories; [α-^{32}P]dATP (6000 Ci/mmol) was from New England Nuclear; nylon membranes (Nytran) were from Schleicher & Schuell. Custom oligonucleotides were synthesized by Pharmacia. Bovine retinal, brain, and lung and rat brain poly(A+) RNA were purified by oligo(dT)–cellulose chromatography from total RNA purified according to the method of Chirgwin et al. (1979). Other preparations of poly(A+) RNA were purchased from

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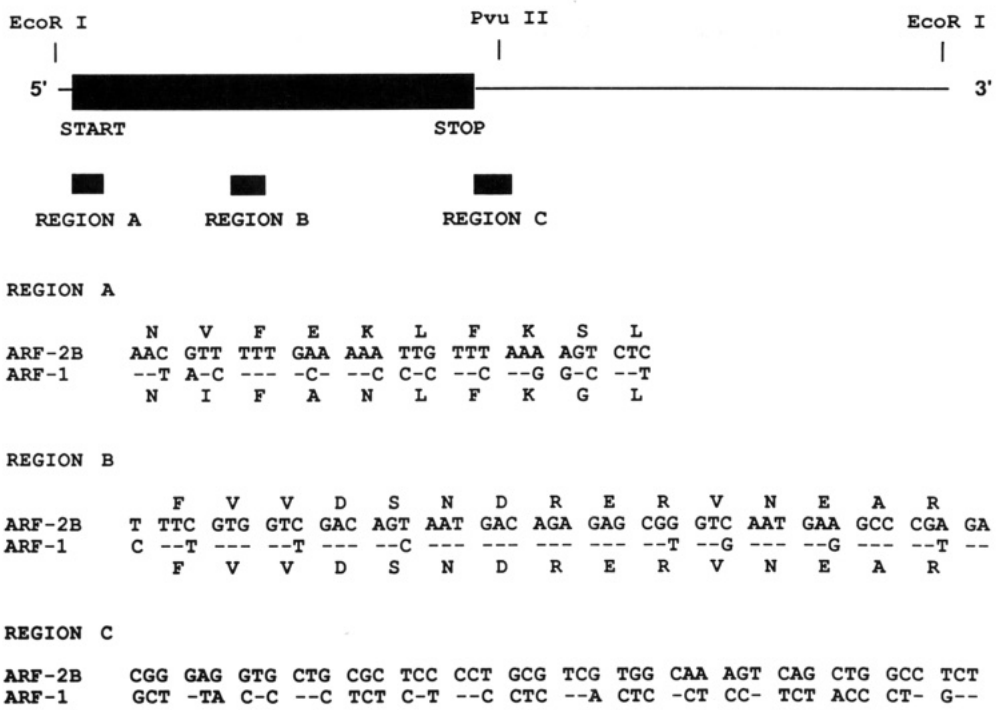

FIGURE 1: Structure of ARF oligonucleotide probes. Shown is a schematic representation of a consensus ARF cDNA in which the darkened bar represents the coding region and the line at the 3′-terminus represents the most proximal untranslated region. Locations of *Eco*RI and *Pvu*II restriction sites in the ARF-2B cDNA (Price et al., 1988) are indicated above; the *Pvu*II restriction site is located 39 nucleotides downstream from the stop codon. Shown below the schematic are nucleotide and deduced amino acid sequences (indicated by their single-letter abbreviation above or below the nucleotide codon) of the bovine retinal (ARF-2B) or adrenal (ARF-1) (Sewell & Kahn, 1988) cDNAs in the regions used as probes. Hyphens in the nucleotide sequence of ARF-1 indicate identity with ARF-2B. *Pvu*II digestion of the ARF-2B cDNA resulted in two cDNAs corresponding primarily to the coding (ARF-2B) and 3′-untranslated (ARF-2Bu) regions (see legend to Figure 11).

Clontech. The bovine retinal ARF cDNA (ARF-2B) and human ARF cDNA (ARF-1) have been described (Price et al., 1988; Bobak et al., 1989b). Human fibroblast cytoplasmic γ-actin cDNA was kindly provided by Dr. Peter Gunning.

Methods. Poly(A+) RNA (10 μg) was fractionated by electrophoresis in 1% agarose/formaldehyde gels and transferred to Nytran. Hybridizations with the [^{32}P]cDNA probe, labeled by the random primer method (Feinberg & Vogelstein, 1983), were performed in 5× SSC/5× Denhardt's solution [1× = 0.02% Ficoll/0.02% polyvinylpyrrolidine/0.02% bovine serum albumin]/10 mM Tris-Cl$^-$, pH 7.4/40% formamide/0.1% SDS/10% dextran sulfate containing denatured salmon sperm DNA (100 μg/mL) at 42 °C for 16 h following prehybridization at 42 °C for 6 h. Filters were washed once with 2× SSC/0.5% SDS and twice with 0.5× SSC/0.5% SDS for 15 min at 55 °C for the ARF cDNA and 65 °C for the γ-actin cDNA. Filters were exposed to Kodak XAR film at −80 °C with intensifying screens. To verify the presence of an equivalent amount of poly(A+) RNA in each lane for all hybridizations, all blots were stripped following hybridization with ARF cDNA or oligonucleotide and hybridized with γ-actin cDNA.

Hybridizations with 48-base oligonucleotides, labeled with [α-^{32}P]dATP by use of terminal deoxynucleotidyl transferase (Eschenfeldt et al., 1987), were performed as described for the cDNA. Filters were washed once with 2× SSC/0.5% SDS and twice with 1× SSC/0.5% SDS at 50 °C. Hybridizations with 30-base oligonucleotides were performed as described for the cDNA except that formamide was omitted from the hybridization buffer. Filters were washed as described for the 48-base oligonucleotides.

Results

Distribution of ARF mRNA in Bovine Tissues. The distribution in bovine tissues of mRNAs related to the two different cDNA clones for ARF was determined by hybridization of poly(A+) RNA with bovine ARF-2B and human ARF-1 cDNAs or two cDNA fragments derived from digestion of ARF-2B cDNA with *Pvu*II and with oligonucleotide probes specific for bovine ARF-2B or ARF-1 (Figure 1). In all tissues examined, the bovine retinal ARF-2B cDNA hybridized predominantly with a 2.1-kb mRNA (Figure 2A; Table I); the human ARF-1 cDNA hybridized with a 1.7-kb mRNA (Figure 3A). An oligonucleotide probe specific for the 5′-end of the coding region of the bovine retinal ARF-2B cDNA hybridized with a 2.1-kb mRNA, whereas a probe specific for

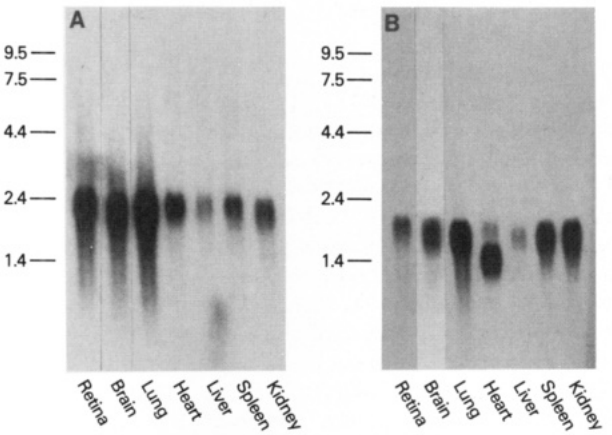

FIGURE 2: Hybridization of poly(A+) RNA from the indicated bovine tissues with (A) retinal ARF-2B or (B) γ-actin cDNA. Positions of RNA standards (nucleotides × 10^{-3}) are indicated on the left. The experiment was repeated three times with similar results. As noted under Methods, γ-actin was used to confirm the presence of an equivalent amount of poly(A+) RNA. In (A), the blot was exposed for 7 days. The specific activity of the probe was 3.7 × 10^9 cpm/μg. In (B), the blot was exposed for 18 h. The specific activity of the probe was 3.4 × 10^9 cpm/μg.

Table I: Mobilities on Agarose Gels of the Major Bovine Tissue mRNA Species That Hybridize with ARF-2B and ARF-1 Probes[a]

tissue	relative sizes (kb) in agarose gels of mRNA hybridizing with probes for ARF-2B	ARF-1
retina	2.32 ± 0.19 (4)	1.90 ± 0.22 (4)
brain	2.10 ± 0.00 (3)	1.68 ± 0.17 (4)
lung	2.12 ± 0.05 (4)	1.75 ± 0.19 (4)
heart	2.13 ± 0.06 (3)	1.87 ± 0.21 (3)
liver	2.10 ± 0.00 (3)	1.80 ± 0.14 (4)
spleen	2.07 ± 0.16 (3)	1.83 ± 0.15 (3)
kidney	1.97 ± 0.12 (3)	1.70 ± 0.20 (4)

[a] Mobilities of the mRNA species were determined relative to the following standards: 9.5, 7.5, 4.4, 2.4, 1.4, and 0.24 kb. The number in parentheses gives the times the mobility was determined; data are expressed as the mean ± standard deviation. The probe for ARF-2B was ARF-2B cDNA, while that for ARF-1 was an oligonucleotide specific for the coding region of bovine ARF-1 cDNA.

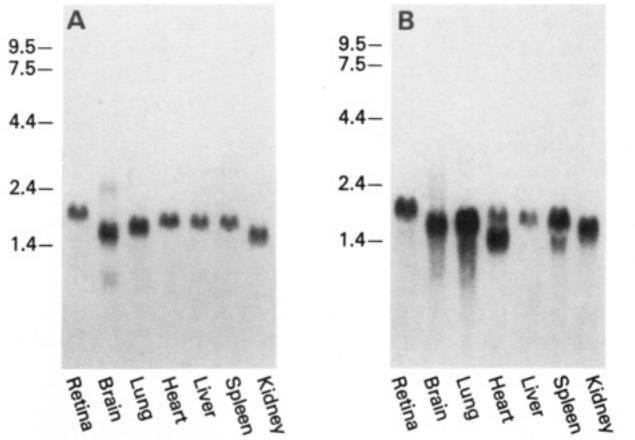

FIGURE 3: Hybridization of poly(A+) RNA from bovine tissues with (A) human ARF-1 or (B) γ-actin cDNA. Positions of RNA standards (nucleotides × 10^{-3}) are indicated on the left. In (A), the blot was exposed for 6 h. The specific activity of the probe was 5.4 × 10^9 cpm/μg. In (B), the blot was exposed for 5 h. The specific activity of the probe was 4.1 × 10^9 cpm/μg.

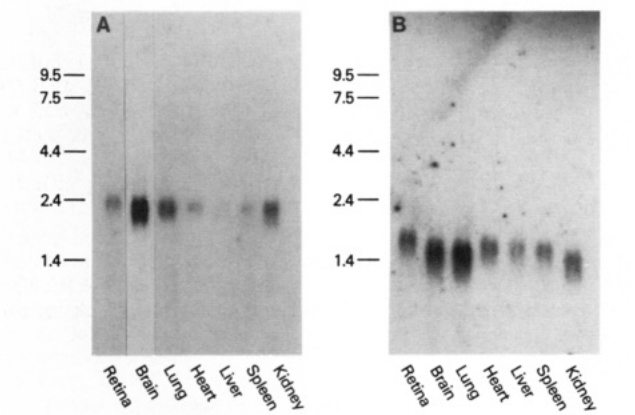

FIGURE 4: Hybridization of poly(A+) RNA from the indicated bovine tissues with oligonucleotides specific for region A (5′-end of the coding region) of bovine (A) retinal ARF-2B or (B) adrenal ARF-1 cDNA. Positions of RNA standards (nucleotides × 10^{-3}) are indicated on the left. The experiment was repeated twice with similar results. In (A), the specific activity of the probe was 8.0 × 10^8 cpm/μg. In (B), the specific activity of the probe was 3.0 × 10^8 cpm/μg. Blots were exposed for 14 days.

the same region of the bovine adrenal ARF-1 hybridized with a 1.7-kb mRNA (Figure 4; Table I). Similarly, an oligonucleotide specific for the 3′-untranslated region of ARF-2B hybridized with a 2.1-kb mRNA, whereas a probe specific for the same region of ARF-1 hybridized with a 1.7-kb mRNA

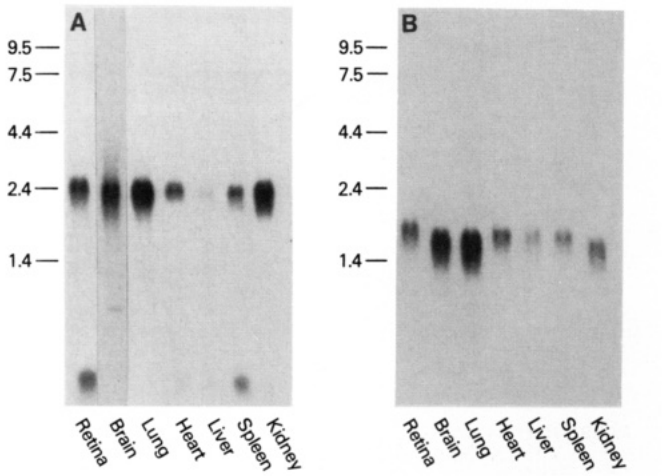

FIGURE 5: Hybridization of poly(A+) RNA from the indicated bovine tissues hybridized with oligonucleotides specific for region C (3′-untranslated region) of bovine (A) retinal ARF-2B or (B) adrenal ARF-1 cDNA. Positions of RNA standards (nucleotides × 10^{-3}) are indicated on the left. The experiment was repeated three times with similar results. In (A), the blot was exposed for 14 days. The specific activity of the probe was 3.5 × 10^9 cpm/μg. In (B), the blot was exposed for 5 h. The specific activity of the probe was 9.0 × 10^8 cpm/μg.

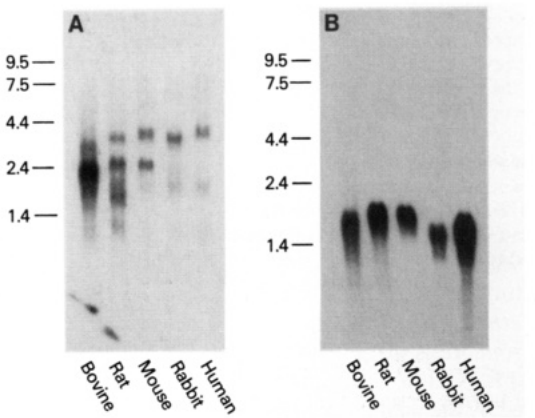

FIGURE 6: Hybridization of brain poly(A+) RNA from the indicated species with (A) ARF-2B cDNA or (B) γ-actin cDNA. Positions of RNA standards (nucleotides × 10^{-3}) are located on the left. The experiment was repeated four times with similar results. In (A), the blot was exposed for 7 days. The specific activity of the probe was 1.2 × 10^{10} cpm/μg. In (B), the blot was exposed for 18 h. The specific activity of the probe was 3.4 × 10^9 cpm/μg.

in all bovine tissues tested (Figure 5; Table I). Thus, on the basis of cDNA sequences and patterns of RNA hybridization with cDNA and oligonucleotide probes, all bovine tissues appear to contain ARF mRNAs of 2.1 and 1.7 kb that are derived from two different ARF genes.

ARF mRNA in Brain from Different Species. Bovine retinal ARF-2B cDNA hybridized with multiple species of brain mRNA from bovine, rat, mouse, rabbit, and human (Figure 6A). Several of these bands are not seen in the hybridization of bovine brain mRNA with ARF-2B cDNA shown in Figure 2A, where probe specific activity was somewhat lower. In contrast, the human ARF-1 cDNA hybridized predominantly to a 1.7-kb mRNA in all species (Figure 7A). To determine which mRNA species were more closely related to the ARF-2B cDNA clone and which to the ARF-1 clone, oligonucleotide probes specific for the coding and noncoding regions of each bovine clone were used. A probe specific for the coding region (region A, Figure 1) of bovine ARF-1 hybridized with 1.7-kb mRNAs from the brain of bovine, rat, mouse, rabbit, and human (Figure 8A; Table II), whereas a

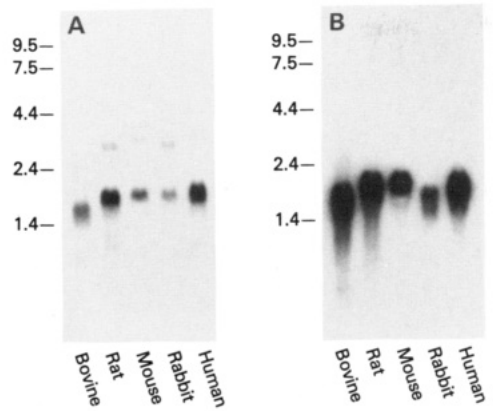

FIGURE 7: Hybridization of brain poly(A+) RNA from the indicated species with (A) human ARF-1 or (B) γ-actin cDNA. Positions of RNA standards (nucleotides × 10^{-3}) are located on the left. In (A), the blot was exposed for 6 h. The specific activity of the probe was 5.4×10^9 cpm/μg. In (B), the blot was exposed for 24 h. The specific activity of the probe was 4.1×10^9 cpm/μg.

Table II: Mobilities on Agarose Gels of the Major Brain mRNA Species That Hybridize with ARF-2B and ARF-1 Probes[a]

source	relative sizes (kb) in agarose gels of mRNA hybridizing with probes for ARF-2B	ARF-1
bovine	2.12 ± 0.08 (7)	1.72 ± 0.05 (7)
rat	2.28 ± 0.15 (7)	1.84 ± 0.08 (7)
mouse	2.33 ± 0.15 (6)	1.83 ± 0.10 (7)
rabbit		1.83 ± 0.10 (4)
human		1.80 ± 0.13 (7)

[a] Mobilities of the mRNA species were determined relative to the following standards: 9.5, 7.5, 4.4, 2.4, 1.4, and 0.24 kb. The number in parentheses gives the times the mobility was determined; data are expressed as the mean ± standard deviation. The probe for ARF-2B was ARF-2B cDNA, while that for ARF-1 was an oligonucleotide specific for the coding region of bovine ARF-1 cDNA.

probe specific for the 3′-untranslated region (region C, Figure 1) of bovine ARF-1, although it hybridized with 1.7-kb mRNA from bovine brain, did not hybridize with mouse, rat, rabbit, or human brain mRNA (Figure 8B). It appears that coding-region sequence is more conserved across species than is that of 3′-untranslated regions.

Oligonucleotide probes derived from the coding (region A, Figure 1) and 3′-untranslated (region C, Figure 1) regions of ARF-2B hybridized with 2.1-kb mRNA from bovine brain but not with brain mRNA from other species (Figure 9). The coding-region oligonucleotide sequence was selected because it differs from the corresponding ARF-1 sequence and could be a relatively specific probe representing a hypervariable region of the protein. An additional coding-region probe, an oligonucleotide representing the midcoding region of the ARF-2B clone (region B, Figure 1), hybridized predominantly with 2.1-kb mRNAs from bovine, rat, mouse, and human brain but not rabbit brain (Figure 10A); an oligonucleotide probe made to the corresponding region of the bovine ARF-1 cDNA hybridized with a 1.7-kb mRNA in all species under low stringency conditions (Figure 10B) but did not hybridize with the 1.7-kb mRNA from rabbit brain under high stringency conditions (Figure 10C).

The restriction enzyme *Pvu*II cuts the ARF-2B cDNA (excised with *Eco*RI) at a single site 39 nucleotides downstream from the stop codon producing two fragments, one representing the coding (ARF-2Bc) and the other the 3′-untranslated (ARF-2Bu) region (900 bases), that were used as probes. The pattern of brain mRNAs that hybridized with probe ARF-2Bc (Figure 11A) was identical with that seen with

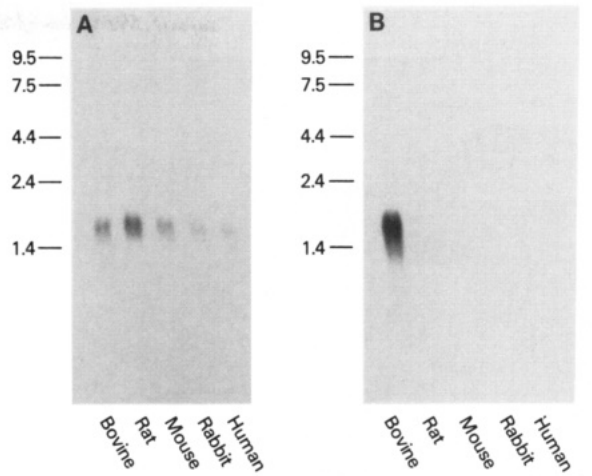

FIGURE 8: Hybridization of brain poly(A+) RNA from the indicated species with oligonucleotides specific for (A) the coding (region A) or (B) the 3′-untranslated region (region C) of the bovine ARF-1 cDNA. Positions of RNA standards (nucleotides × 10^{-3}) are located on the left. The experiment was repeated three times with similar results. In (A), the specific activity of the probe was 2.9×10^9 cpm/μg. In (B), the specific activity of the probe was 1.7×10^9 cpm/μg. Blots were exposed for 18 h.

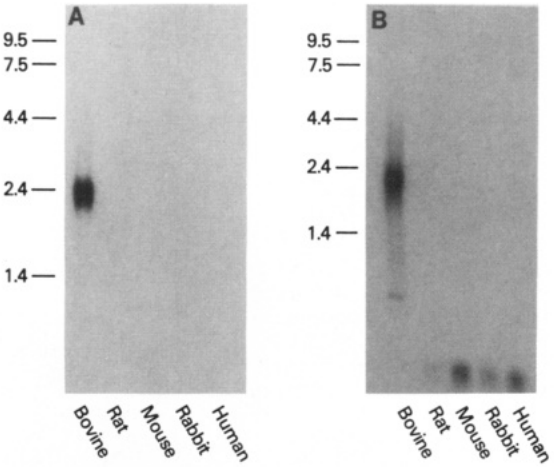

FIGURE 9: Hybridization of brain poly(A+) RNA from the indicated species with oligonucleotides specific for (A) the coding (region A) or (B) the 3′-untranslated region (region C) of ARF-2B cDNA. Positions of RNA standards (nucleotides × 10^{-3}) are located on the left. The experiment was repeated twice with similar results. In (A), the specific activity of the probe was 2.3×10^9 cpm/μg. In (B), the specific activity of the probe was 3.5×10^9 cpm/μg. Blots were exposed for 14 days.

the intact ARF-2B cDNA. Probe ARF-2Bu, however, hybridized with only the 2.1-kb mRNA from bovine brain (Figure 11B).

Discussion

By use of cDNA and oligonucleotide probes, two different ARF mRNAs were found in all bovine tissues examined, a 2.1-kb species that hybridized exclusively with the ARF-2B cDNA or 2B-specific probes and a 1.7-kb species that hybridized with the human ARF-1 cDNA or probes specific for bovine ARF-1. All observations are consistent with the conclusion that the 2.1- and 1.7-kb mRNAs are the products of two different genes. That mRNA other than the 2.1- and 1.7-kb species hybridized with the bovine ARF-2B and human ARF-1 cDNAs as well as an oligonucleotide representing the midcoding region of the bovine ARF-1 cDNA in brain from different species suggests that there may be other ARF-related gene products.

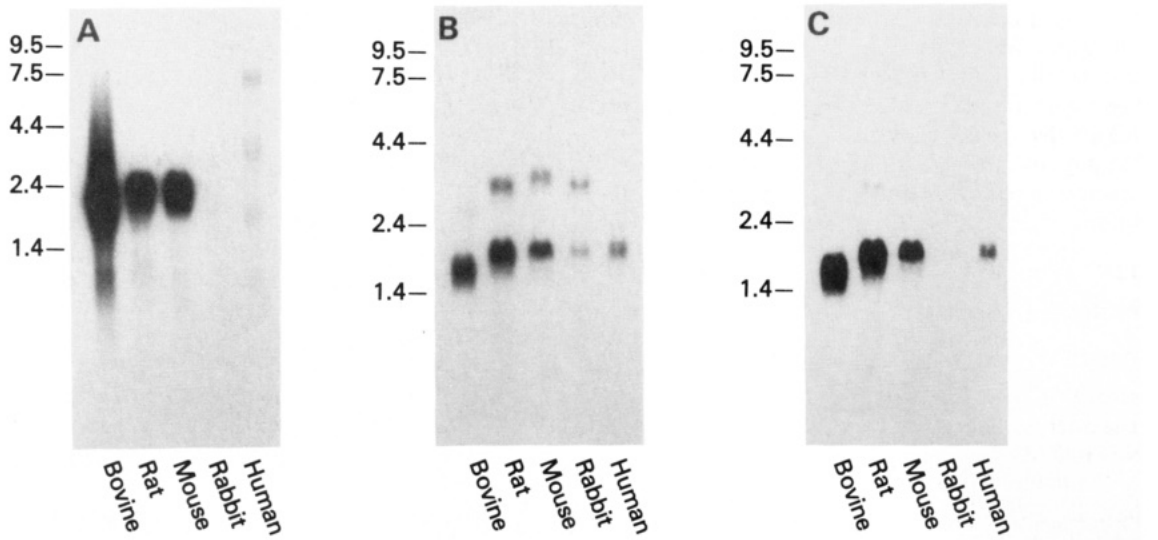

FIGURE 10: Hybridization of brain poly(A+) RNA from the indicated species with an oligonucleotide specific for the coding region (region B) of the bovine retinal ARF-2B cDNA (A) or adrenal ARF-1 cDNA (B and C). Positions of RNA standards (nucleotides $\times 10^{-3}$) are located on the left. The experiment was repeated twice with similar results. In (A), the blot was exposed for 11 days. The specific activity of the probe was 3.3×10^9 cpm/μg. In (B) and (C), the blot was washed at 50 °C with 1× SSC/0.5 SDS and exposed for 6 h (B), followed by washing at 60 °C with 0.1× SSC/0.1 SDS and exposure for 18 h (C). The specific activity of the probe was 1.5×10^9 cpm/μg.

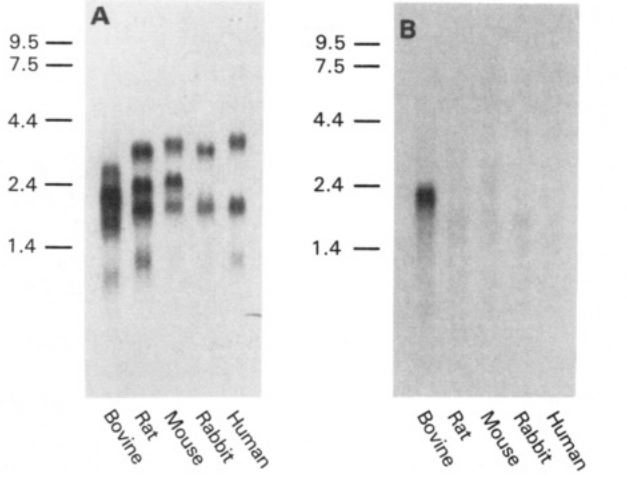

FIGURE 11: Hybridization of brain poly(A+) RNA from the indicated species with restriction fragments of ARF-2B cDNA representing (A) coding or (B) 3′-untranslated regions. cDNA probes used for hybridization were (A) an *Eco*RI–*Pvu*II restriction fragment (bases −7 to 592) containing the complete coding region and (B) a *Pvu*II–*Eco*RI fragment (base 593 to 3′-terminus of the cDNA, ~900 bases) encompassing only 3′-untranslated region of the ARF-2B cDNA. Positions of RNA standards (nucleotides $\times 10^{-3}$) are indicated on the left. In (A), the blot was exposed for 18 h. The specific activity of the probe was 1.2×10^{10} cpm/μg. In (B), the blot was exposed for 18 h. The specific activity of the probe was 1.3×10^{10} cpm/μg.

Two soluble and one membrane form of ARF have been purified from bovine brain. The partial amino acid sequence obtained from CNBr peptides from the membrane ARF (Kahn et al., 1988) is not identical with deduced amino acid sequences from ARF-1 or ARF-2B cDNA. The partial amino acid sequence of sARF-II (Price et al., 1988), one of the soluble ARFs, matches exactly the deduced sequence from ARF-1 cDNA (Sewell & Kahn, 1988) and differs in 2 of 60 positions from the deduced sequence of the ARF-2B cDNA; available data are consistent with the possibility that the ARF-1 cDNA represents the message for sARF-II. The protein that corresponds to the ARF-2B cDNA remains to be identified. It could be sARF-I (Tsai et al., 1988a), for which no amino acid sequence is available, or there may be other ARF proteins that have not been characterized.

In their coding regions, the bovine ARF-2B and ARF-1 cDNAs are 80% identical; as most differences are in the third position of the codon, the deduced amino acid sequences are 96% identical. It remains to be determined how the differences, which are chiefly near the carboxy and amino termini of the protein, affect function or biological activity. Oligonucleotide probes representing the 5′-ends of the coding regions of the cDNAs were used to assist in discriminating between mRNAs derived from the two ARF genes. The fact that the oligonucleotide specific for the bovine ARF-1 cDNA hybridized with mRNAs of 1.7 kb from bovine, mouse, rat, human, and rabbit brain is consistent with a high degree of conservation of ARF-1. An analogous coding-region probe specific for the bovine ARF-2B cDNA did not hybridize with mRNA from brain of other species nor did oligonucleotides specific for 3′-untranslated regions of either of the two bovine ARF cDNAs, suggesting that the 3′-untranslated regions are more divergent. In agreement, of the *Pvu*II fragments of ARF-2B cDNA, only that containing the coding region hybridized with mRNA from brain other than bovine. Thus, the coding regions of ARF-1 and ARF-2B appear to be more extensively conserved across species than are the 3′-untranslated regions of either cDNA. In this regard the ARF family of cDNAs may be different from those for the 40-kDa α-subunits of guanine nucleotide binding (G) proteins that participate in receptor-mediated signal transduction in which both coding regions and 3′-untranslated regions, at least those portions adjacent to coding regions, appear to be highly conserved across species (Bray et al., 1987; Lochrie & Simon, 1988).

Although the ARF proteins are apparently relatively ubiquitous, their function in animal cells is unclear. There is, at present, no evidence of any functional relationship between ARF, or ARF-like 19–24-kDa guanine nucleotide binding proteins, and the G proteins involved in transmembrane signaling. Data from several laboratories are consistent, however, with a role for some of these small guanine nucleotide binding proteins in intracellular transport, i.e., endoplasmic reticulum–Golgi–plasma membrane (Melancon et al., 1987; Bourne, 1988; Goud et al., 1988; Segev et al., 1988). The 21-kDa ras proteins are involved in cell growth and transformation (Barbacid, 1987). All of these proteins share similarities in deduced amino acid sequences in regions believed to be in-

volved in guanine nucleotide binding and hydrolysis (Chardin & Tavitian, 1986; Haubruck et al., 1987; Touchot et al., 1987; Chardin et al., 1988; Kawata et al., 1988; Pizon et al., 1988; Kitayama et al., 1989). The other regions of the proteins, which are divergent, are presumably important for their different physiological roles. The availability of cDNA clones for many of these proteins may facilitate identification of their functions and the structural bases of functional specificity.

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