A new member of the ras gene superfamily identified in a rat liver cell line

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Received August 26, 1988; Accepted October 3, 1988

ABSTRACT
A new member of the ras genes superfamily was isolated from a cDNA library derived from a rat liver cell line (BRL-3A). The predicted 201 amino acids ras-like protein shows 30-35% homology with other members of the ras and ras-related gene products so far described. Conserved features include the GTP-binding and hydrolysis domains and the carboxyl terminal cysteine residues. A protein of the expected size (Mr 23,000) was synthesized in an in vitro transcription-translation system. The BRL-ras gene is present in single copy in the rat genome and is ubiquitously expressed at high levels in all tissues and cell lines examined.

INTRODUCTION
Recent evidence which has accumulated in the past few years has indicated that ras and ras-related genes code for a superfamily of similar proteins sharing limited (30-50%) overall homology (1). This homology is usually due to the preservation of a few defined domains, in the proteins, which have been shown to be involved in the binding and hydrolysis of GTP (1-4). Members belonging to the same family, as well as homologous counterparts from different species even quite removed evolutionary, share a much higher degree of similarity and possibly have comparable functions (5).

Some of these genes were fortuitously discovered (6,7) and an high number of ras-related genes was isolated through screening of cDNA and genomic libraries, either with synthetic oligonucleotides corresponding to conserved domains or by using low stringency hybridization conditions (8-11). This fact suggests that the ras genes superfamily might comprise many more members than those currently identified. Although the physiological function of these proteins is still poorly
understood it is reasonable to assume that the large number of different, but structurally related, guanine nucleotide-binding proteins, might regulate various cellular activities.

In the present work we report the identification and characterization in rat of a novel ras-related gene (BRL-ras) which appears to encode for a member of the family of the ras-like proteins and which is transcribed with high efficiency at different ages and in many tissues.

MATERIALS AND METHODS

Chemicals and enzymes

Restriction endonucleases, DNA polymerases, T4 DNA ligase, T4 polynucleotide kinase, reverse transcriptase and other enzymes were purchased from commercial suppliers (Boehringer Mannheim, Promega, Amersham Corp. and Bethesda Research Laboratories). All deoxynucleotide triphosphates, dideoxynucleotide triphosphates and ribonucleotide triphosphates were from Pharmacia and all radionucleides were obtained from Amersham Corp.

Isolation of cDNA clones

A cDNA library was constructed from 12S poly(A)⁺ RNA from BRL-3A cells using the procedure of Land et al. (12) with modifications, as previously described (13). The resulting tetracycline resistant clones were screened using a mixture of tetradecamers 5'-d(AARCARCAYTCYTC)-3' that have been 5' labeled with T4 polynucleotide kinase and [γ-³²P]ATP.

An additional cDNA library was constructed from BRL-3A polyadenylated RNA. Complementary DNA was prepared with a cDNA synthesis kit (Amersham Corp.) following recommended procedures, except that methylation of double stranded cDNA before the addition of linkers was omitted. The λgt10 vector was cleaved with restriction endonuclease EcoRI and the cDNA was ligated to the phage arms after addition of EcoRI linkers. Recombinant phage DNA was packaged in vitro using the Gigapack kit supplied by Stratagene. The library was screened by the in situ plaque hybridization technique (14).

A cDNA 650 bp PstI restriction fragment was labeled to a specific activity of 2x10⁹ cpm/µg and used as a probe to screen the library. The filters were washed under stringent conditions.
(0.1 X SSC, 0.1% SDS, 65°C). Clones which gave a positive signal on duplicate filters were purified through three consecutive cycles and phage DNA was isolated by a small scale purification procedure (14).

Restriction maps and DNA sequence

Restriction maps were determined by single and double digestion on the plasmid or on the entire phage DNA. Restriction fragments were purified by electrophoresis in 4%-6% acrylamide gels and then phenol extracted and ethanol precipitated. DNA sequencing was performed either according to Maxam and Gilbert (15) or by the dideoxy chain terminating method (16) after subcloning restriction fragments into M13 vectors (17). Data analysis was simplified by the use of the computer program Microgenie (Beckman) run on an IBM AT computer.

Nucleic Acids Preparations

RNA was isolated from cultured cells or from rat tissues either by extraction with guanidinium thiocyanate (18) or by using the guanidinium thiocyanate (Fluka)/LiCl precipitation procedure (19). Polyadenylated RNA was isolated by oligo (dT)-cellulose chromatography. RNAs were electrophoresed on 1.2% agarose gels containing 2.2 M formaldehyde (20). After electrophoresis the gels were blotted without further treatment and hybridized to labeled probes.

Genomic DNA was extracted from BRL-3A and BRL-3A2 cell lines and from rat liver tissue and after digestion with different enzymes was electrophoresed on agarose gels (0.8%) in either TBE or TAE buffers (14) and then blotted according to the method of Southern (21). Plasmid DNAs were purified according to the procedure of Clewell (22) and were finally banded on a cesium chloride ethydium bromide equilibrium density gradient. Cell transformations were performed with the standard CaCl₂ technique (23).

Labeling of DNA

Radioactive probes for hybridization experiments were prepared either by conventional nick translation (specific activity: 2 x 10⁸ cpm/μg) (24) or by utilizing the random priming DNA labeling technique (specific activity: 2-3 x 10⁹ cpm/μg) (25).
Blots were hybridized at 42°C in 50% formamide, 6X SSC, 10X Denhardt's, 100 μg/ml sheared denatured salmon sperm DNA, 0.5% SDS. Southern blots were washed at stringent conditions (0.1X SSC, 0.1% SDS, 65°C). Northern blots were washed at 42°C, 0.2X SSC, 0.1% SDS.

Protein synthesis

A BRL-ras 5' proximal HincII-EcoRI 580 bp cDNA restriction fragment was isolated from plasmid pR1 and cloned in vector plasmid pUC12 (26) to generate recombinant plasmid pR12. Then the 650 bp EcoRI fragment, corresponding to the 3' distal BRL-ras region, was isolated from recombinant clone XR8 and inserted in plasmid pR12 cleaved with EcoRI. The correct orientation was verified by digestion with BglII possessing two asymmetric cleavage sites within the inserts. The resulting recombinant plasmid pR13 was cut with the restriction enzyme HindIII and the 1 Kb HindIII restriction fragment, containing the BRL-ras open reading frame was isolated and cloned in the pGem-3 plasmid (27) under the SP6 promoter. A similar recombinant plasmid was also constructed in vector pGem-4 and contained the complete coding and 3' flanking regions of the rIGF-II pre-pro peptide in a cDNA EcoRI fragment of 850 bp. The linearized plasmid DNAs were then used in an in vitro transcription system (Riboprobe SP6 RNA Polymerase, Promega) and the RNAs synthetized were isolated and used in an in vitro translation system (Promega, Lysate Nuclease Treated, Rabbit Reticulocyte) using [35S]-labeled methionine. The proteins were electrophoresed in 15% SDS polyacrylamide denaturing gels (28) at 15 mA overnight. The gels were prepared for fluorography (29), dried under vacuum and exposed to Kodak X-Omat AR films at -80°C with intensifying screens.

RESULTS

A new ras-related gene is expressed in the BRL-3A rat liver cell line

During the screening of a cDNA library, derived from poly(A)⁺ RNA from a Buffalo rat liver cell line (BRL-3A) (30), with a synthetic tetradecamer oligonucleotide derived from the insulin-like growth factor II (rIGF-II) amino acid sequence, we isolated and sequenced several clones that were found not to correspond to the rIGF-II sequences (13). Among them two shared
Fig. 1. Restriction map, cDNA clones and DNA sequence of BRL-ras. 
A. Combined restriction map (Top) of the cDNA fragments (open boxes) cloned in recombinant plasmids pR1 and λR8 (Bottom). Only the restriction sites which were used for construction of subclones and for isolation of probes are shown. Hc = HindIII, Bg = BglII, B = BamHI, E = EcoRI, P = PstI, S = SacI, H = HindIII, P = Cloning PstI site lost. Middle: Overall organization 5' to 3' of the BRL-ras cDNA sequence. Hatched box = coding sequences; solid box = poly(CA) repetitive element.
B. Composite DNA sequence of the two cDNAs. Amino acids corresponding to the putative ORF are indicated below the sequence in the single letter code. The nucleotides corresponding to the poly(CA) element are underlined.
common regions and, therefore, appeared to be derived from a major species of mRNA present in this cell line. A search against the nucleotide database (Gene Bank) detected a significant homology, over 60% in a stretch of about 150 nucleotides, with several ras and ras-related genes (data not shown). The longest of the two clones, pR1 (Fig. 1A), contained a cDNA insert of 700 bp. When this cDNA was used as a probe on Northern blots, mRNA species up to 2.5 kb were detected (see below). We therefore screened another cDNA library derived from the same cell line. The new library was screened with a PstI 650 bp fragment derived

Fig. 2. Alignment of the BRL-ras protein sequence (middle) with the RAT H-ras-1 (top) and RAT rab-1 (bottom) proteins. The amino acids are indicated by the single letter code. Gaps were introduced for optimal alignment. Sets of identical (asterisks) or conservative (colons) residues are indicated. Conserved GTP-binding and hydrolysis domains and the carboxyl terminal cysteine domain are boxed. The H-ras-1 Gly 12 and the equivalent Ser residues in BRL-ras and rab-1, as well as the effector (residues 30-42) and antibody- binding (residues 63-73) domains of H-ras-1 are underlined. The reference numbering is that of H-ras-1.
from plasmid pRl and a large number of positive clones were isolated, again suggesting that these mRNAs are abundantly represented in BRL-3A cells. Some of these clones were purified and one, 680 bp long (λR8), was further characterized (Fig. 1A). The DNA sequence derived from the two overlapping clones pRl and λR8 is 1268 bp long and is shown in Fig. 1B. The sequence does not contain a canonical polyadenylation site (31) suggesting that the mRNAs extend further in the 3' direction. Within the sequence there is a short stretch of poly(CA), nucleotides 795-842 (Fig. 1B). Such sequences have been identified as repetitive elements (32) and have been found at the 3' or 5' end of several mRNAs (33-36). These elements can assume a left-handed DNA conformation and function as "enhancers" in different cells (37).

![Diagram](image)

**Fig. 3.** Comparison of the conserved domains and hydropathy profiles of BRL-*ras* (a), RAT *H-ras*-1 (b) and RAT *rab*-1 (c).

Top: The functionally relevant structural domains involved in GTP-binding and hydrolysis are indicated by open boxes. The rat *H-ras*-1 effector domain (residues 30-43) by a solid box and the antibody-binding domain (residues 30-43) by a hatched box.

Bottom: In the hydropathy profiles the positive values indicate hydrophylic regions and negative values hydrophobic segments. The hydropathic value of each amino acid residue is plotted from the amino terminus to the carboxyl terminus (left to right along the abscissa).
Analysis of the entire cDNA sequence for potential coding regions showed the presence of only one long open reading frame (ORF), nucleotides 59-664. The 201 amino acids long sequence of this ORF is shown below the cDNA sequence in Fig. 1B. This putative protein has been compared with many different members of the ras genes superfamily and in each case a significant degree of homology, over 30%, was found. The alignment of the BRL-ras protein with two representative members, Rat H-ras-1 (38) and Rat rab-1 (9), is shown in Fig. 2.

Expression of the BRL-ras gene

To determine the relative abundance and the size of the mRNA(s) corresponding to our cDNA we performed a Northern blot with BRL-3A total and poly(A)+ RNA using as a probe a 650 bp PstI DNA fragment spanning the coding region (Fig. 4A). One major species of 2.5 kb and a minor one of 1.5 kb were detected. The strength of the signal and a comparison with IGF-II mRNAs which are also abundantly expressed in this cell line (40) (data not shown) again seem to indicate that these mRNAs are present in large amounts. To rule out possible cross hybridization with other ras mRNAs, and to determine whether the expression is confined only to the BRL-3A cell line, we performed Northern blots with total RNA isolated from other cell lines and from one tissue using as a probe a 3'-specific 310 bp HindIII-EcoRI fragment. The same two mRNA species and comparable steady-state levels of expression were detected, not only in the BRL-3A cell line and in two different subclones derived from this same line, BRL-3A2 and BRL-30E (30) (Fig. 4B), but also in the L6 mioblast cell line (41) and in rat kidney tissue (Fig. 4C).

These findings indicate that the expression of the BRL-ras-related gene is not confined to a single cell type, but that the gene is transcribed in various cells and tissues. We have extended the transcriptional analysis by performing Northern
blots on RNAs extracted from thirteen rat tissues collected at different developmental stages (fetal and adult). The same two mRNAs species were detected irrespective of the tissue or age examined. The data obtained from six tissues (liver, kidney, stomach, heart, muscle and hypothalamus) and from three developmental ages (21 days fetal, 21 and 75 days adult) are presented in Fig. 5. These data establish that expression of the BRL-ras gene is constitutive, very efficient and ubiquitous.

We next wished to determine whether the mRNA heterogeneity and the high levels of expression were due to the presence of different alleles and/or multiple copies of the gene. We performed Southern blots of genomic DNA extracted from various rat cell lines and liver tissue and digested with several restriction enzymes using as a probe a cDNA HincII-SacI 750 bp fragment (Fig. 1A) spanning the entire coding sequence and part of the 3' untranslated region with the exception of the repetitive poly(CA) element. In every instance only the number of bands predicted from the restriction map of the cDNA was observed. These data indicate that BRL-ras is a single gene. In

Fig. 4. Northern blots of BRL-ras RNA.
Panel A. RNA extracted from BRL-3A cells hybridized to a cDNA coding fragment. Lane 1: total RNA (10 μg); lane 2: poly(A)⁺ RNA (3 μg). Panel B. Total RNA (10 μg) extracted from BRL-3A cells (lane 1); BRL-3A2 cells (lane 2); BRL-30E cells (lane 3) hybridized to a 3' distal fragment. Panel C. Total RNA (10 μg) extracted from L6 cells (lane 1); from rat kidney tissue (lane 2) and hybridized to the same probe used in Panel B. The autoradiogram shown in Panel A was exposed for 4 hours and those shown in Panel B and C for 12 hours. The relative migration of the ribosomal RNAs (28S and 18S) is indicated on the left of each panel.
Fig. 5. Distribution of BRL-ras RNA in different fetal and adult rat tissues.

Northern blots of total RNA (15 μg/lane) isolated from the following fetal and adult rat tissues: Liver (Panel A); Kidney (Panel B); Stomach (Panel C); Heart (Panel D); Muscle (Panel E); Hypothalamus (Panel F). The blots were hybridized with a 650 bp PstI cDNA fragment of the coding region of BRL-ras. RNAs were extracted from foetuses of 21 days (21f) or from adult animals of 21 or 75 days (21d, 75d). The autoradiograms were exposed for 4 hours. The relative migration of the ribosomal RNAs (28S and 18S) is indicated on the left.

addition titration experiments with different amounts of recombinant pRl plasmid DNA also indicate that the BRL-ras gene is present in single copy in the genome (data not shown).

The 201 amino acids long putative protein has an estimated Mr of 22,800. To obtain independent evidence that the DNA sequence was correct and that such a protein is actually encoded in our cDNAs we performed in vitro transcription-translation experiments. An expression vector containing the coding region was constructed in the pGem system (27) as detailed in Materials and Methods. This DNA was transcribed with SP6 polymerase and the RNA was isolated and translated in a reticulocyte lysate system. Analysis of the labeled proteins in SDS polyacrylamide gels showed that a protein of the expected size was very efficiently
Fig. 6. Analysis of \textit{in vitro} translation products on SDS-polyacrylamide gels.

Aliquots of translation incubations containing RNA transcribed \textit{in vitro} with SP6 polymerase from linearized vector pGem-3 (lane 2); recombinant vector Gem-BRL-ras (lane 3) and recombinant vector Gem-rIGF-II (lane 4) were electrophoresed on 15% SDS-polyacrylamide gels, treated and fluorographed. Exposure time was for 6 hours. Relative migration of $^{14}$C-labeled molecular weight markers is shown in lane 1 and the molecular weights are indicated on the left.

synthesized (Fig. 6, lane 3). The result obtained using an analogous vector, containing the coding region for Mr 20,100 rat pre-pro-IGF-II protein (42, 43), is shown for comparison in Fig. 6, lane 4.

DISCUSSION

We have characterized a new member of the \textit{ras} genes superfamily which appears to represent a novel member of the \textit{ras}-related genes. The evidence so far obtained have established that this superfamily of genes is composed of families like the \textit{ras} and \textit{rho} which comprise several members closely related to each other and which are highly conserved throughout evolution (reviewed in 1). In addition many members have been identified in several species which exhibit limited homology to the \textit{ras} and \textit{rho} families and among themselves, mostly confined to the GTP-binding and hydrolysis domains. They include the yeast \textit{YPT1} (44) and its mouse \textit{yptl} (45) and rat \textit{rabl} (9) counterparts; the simian \textit{ral}
(8); the human and mouse R-ras (10); the rat rab2, rab3 and rab4 (9) and the yeast SEC4 (46).

The features of the BRL-ras gene product closely resemble those of the other ras-related proteins. In fact it has a rather low overall homology (30-35%) with all the members previously discussed. The similarities are mostly confined to the four domains in the proteins which have been shown to participate in the binding and hydrolysis of GTP (reviewed in 1) (Fig. 2). A more detailed analysis of the comparison between the primary amino acid sequence and hydropathy profile of BRL-ras and that of two representative members (H-ras-1 and rab-1, Fig. 2 and Fig. 3) shows several interesting characteristics. (i): BRL-ras has a Ser residue in position 11, equivalent to Ser 12 of the activated v-rasH gene (47), whereas the cellular counterpart has Gly in that position (38). In this respect BRL-ras resembles the Ypt-rab members which also have Ser at the equivalent position (5, 9). (ii): Within the carboxyl-terminal hypervariable domain (residues beyond 165) BRL-ras has two alternate Cys residues at the carboxyl-terminus and therefore in this respect is similar to rab-3 and rab-4 (9). In ras it has been shown that the Cys residue at position 186 is essential for the transforming activity (48) and is required for post-translational modification with palmitic acid for membrane anchorage (49). The variations in the structural organization of this domain might reflect a different subcellular localization of these proteins. It has in fact been shown that yeast and mouse ypt-proteins, which possess tandem carboxyl-terminal cysteine residues, are localized to the Golgi apparatus (5). (iii): The primary amino acid sequence as well as the hydropathic profile of the so called effector domain (residues 30-43) (1) are rather different in BRL-ras compared to real ras and more similar to members of the Ypt-rab family. This effector domain has been very recently identified as the region of ras protein that binds the cytoplasmic protein GAP (50) implicating this factor as the biological target for regulation by p21 (51). Another limited region of divergence is the one from residue 63 to 85, spanning the Y13-259 monoclonal antibody binding domain of ras proteins (52). This region is strongly hydrophilic in ras and hydrofobic in BRL-ras and much more
similar to the rab members except for the presence of two unique Cys residues at position 77-78. It has been suggested that this region might interact with a family of rab-specific regulatory proteins (9).

We have shown that BRL-ras is transcribed into two mRNA species, a major one of 2.5 and a minor one of 1.5 kb (Figs. 4 and 5) and that the very abundant steady state levels of these transcripts are not due to multiple alleles or to gene amplification. The pattern of expression is similar in many cell lines exhibiting quite distinct phenotypes and in various tissues at different developmental stages.

The transcription pattern of real ras genes is quite different from that of BRL-ras. In fact they are transcribed at low levels and are preferentially expressed in some cell lineages, suggesting a role not only in growth control but also in terminal differentiation (1). On the other hand some ras-related genes, like the Aplysia rho (6) and mouse Ypt1 (45) have a pattern of expression more similar to the one we found for BRL-ras.

The physiological role of most ras and ras-related gene products is still very poorly understood. An exception are some proteins that have been identified and characterized in yeast. Thanks to the availability of spontaneous mutants and by in vitro mutagenesis experiments it has been possible to understand the function of factors like RAS1 and RAS2 (11), SEC4 (46) and YPT1 (44). For instance YPT1 is an essential factor which is required for microtubule organization, cell division, and protein secretion, possibly by regulating the intracellular calcium levels (2, 5, 53, 54). We have no clues at the moment on the cellular functions and subcellular localization of this new ras-related gene product, but in view of its peculiar features it is likely to be involved in some process of signal transduction through transmembrane signaling systems (55).

ACKNOWLEDGEMENTS

This work was partially supported by funds from Progetto Finalizzato Oncologia of the C.N.R.
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