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Genetics

A conserved retina-specific gene encodes a basic motif/leucine zipper domain

(subtraction cloning/DNA binding protein/v-*maf* oncogene/signal transduction/evolution)

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ABSTRACT **Using subtraction cloning, we have isolated a human cDNA, AS321, which is expressed in retina and retinoblastoma cell lines but not in any other tissue or cell line tested. AS321 mRNA is detected in all cells of neural retina, with a high level of expression in photoreceptors. The polypeptide sequence deduced from the cDNA reveals consensus phosphorylation sites for protein kinase A and proline-directed protein kinase. Its C-terminal region contains a basic motif and a leucine zipper domain similar to the DNA binding proteins of the jun and fos oncoprotein family, and it shows a strong similarity to the product of an avian retroviral oncogene, v-*maf*. The gene for AS321 is conserved during evolution and is expressed in vertebrate retina. We propose to name the gene NRL (neural retina leucine zipper).**

Light initiates a cascade of biochemical events in retina where visual information is collected, processed, and then transmitted by highly specialized and diverse neuronal cell types. The development of neural retina is a result of highly coordinated expression of specific genes and their products, as reflected by the large array of developmental and hereditary diseases affecting the visual system (1, 2). A number of photoreceptor-specific genes encoding components of the phototransduction cascade have recently been isolated, and some of the early steps in the chain of biochemical events triggered in response to light are becoming clear (3–9). However, the molecular nature of neuronal differentiation and of information processing by distinct retinal cell types is not understood.

Differentiation of a cellular phenotype is often a result of positive and negative control mechanisms influencing the pattern of gene expression in a stage- and cell type-specific manner (10). In higher eukaryotes, a major regulatory function during cell proliferation and differentiation is exerted by the sequence-specific DNA binding proteins (11–13). Interaction of specific regulatory proteins with other components of the transcription initiation complex determines the pattern of gene expression. *jun* and *fos* define a family of "early response" genes that are expressed in a number of tissues/cell types and respond to stimuli by altering the programs of cell growth and differentiation (14–16). The members of this family encode nuclear transcription factors that possess a leucine zipper domain preceded by a basic region and can form heterodimeric complexes with each other. We report here the identification of a human cDNA, AS321, which is expressed specifically in neural retina and encodes a polypeptide of the jun/fos oncoprotein family.¶ The evolutionary conservation of the gene for AS321 suggests a significant function for its product in retinal development and/or signal transduction.

MATERIALS AND METHODS

Materials. Human tissues were obtained from National Disease Research Interchange, Philadelphia. Genomic DNAs for Zoo blot analysis (Southern analysis of DNA from various organisms) and *in vitro* translation kits were purchased from Promega. HeLa nuclear extract and the oligonucleotides used for DNA binding studies were part of a gel shift assay kit (Stratagene). A Northern blot of RNA from cancer cell lines was kindly provided by Lone Anderson (University of Michigan, Ann Arbor). The mouse c-*fos* and c-*jun* cDNAs in T7 promoter-containing vectors and antisera against their products were from Inder Verma's laboratory (Salk Institute, San Diego).

Methods. The methods for preparation and analysis of nucleic acids, construction of cDNA libraries, *in vitro* translation, and *in situ* hybridization to tissue sections have been described (17, 18). Poly(A)$^+$ RNA from a few tissues/cell lines was isolated by using a FastTrack kit (InVitrogen, San Diego). DNA and protein sequence analyses were performed with MacVector (IBI). A similarity search was done against the GenBank data base (version 65). Protein association and gel shift analyses were performed according to published methods (19).

RESULTS

Isolation and Expression of AS321 cDNA. To identify the genes involved in retinal function, we constructed a cDNA library from human adult retina and enriched it for specific genes by an efficient biotin-based subtraction procedure (20). Northern analysis with AS321, a randomly picked clone from the subtracted retina library, showed a major 1.3-kilobase (kb) and two minor 2.0- and 4-kb transcripts only in retinal RNA from various developmental stages and in retinoblastoma cell lines (Fig. 1). The transcripts were not detected in any other human tissue or cell line tested. To localize the mRNA in specific cell layers of retina, *in situ* hybridization was performed with sections of adult baboon retina. AS321 mRNA was present in all cells of neural retina, including the outer (photoreceptor cells) and inner nuclear layers (bipolar, horizontal, and amacrine cells) of retina (Fig. 2). The ganglion cells also showed a signal above background. Similar results were obtained with mouse retina sections (unpublished data).

Analysis of cDNA Sequence and Deduced Polypeptide. To obtain full-length cDNA clones and to identify the nature of larger transcripts, 11 additional clones were isolated from a

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. M81840).

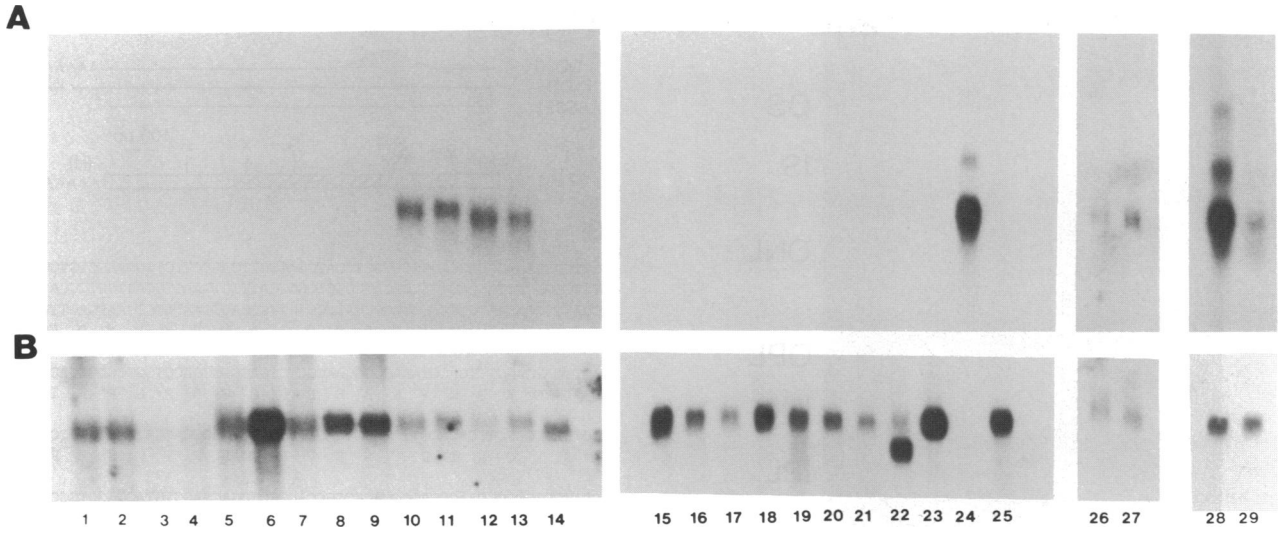


FIG. 1. Expression of AS321 mRNA. Northern blots of total (8–10 μg; lanes 1–14, 26, and 27) or poly(A)$^+$ RNA (2–3 μg; lanes 15–23, 25, 28, and 29; <1 μg, lane 24) were hybridized with ^{32}P-labeled AS321 cDNA (*A*) (base pairs 24–989; see Fig. 3) or β-actin (*B*) and washed under high stringency. All RNAs were from human tissues or cell lines except for those in lanes 26 and 27. Lanes: 1 and 20, fetus + placenta (9 wk); 2, brain (75 yr); 3, kidney (2 mo); 4, liver (2 day); 5, thymus (21 yr); 6 and 23, JY lymphoblastoid cell line; 7, lung (newborn); 8, fetal eye + brain (10.5 wk); 9, retinal pigment epithelium cell line; 10–13 and 24, retina (14 mo, 15 yr, 30 yr, 61 yr, 16 yr); 14 and 21, fetal thymus (26 wk); 15, HeLa cell line; 16, NTera 2/D1 teratocarcinoma cell line; 17 and 18, fetal brain (23 and 11 wk); 19, fetal eye (11 wk); 22, muscle (newborn); 25, primary retinal pigment epithelial cells; 26, fetal baboon retina (140 days); 27, adult baboon retina; 28 and 29, Y79 and WERI retinoblastoma cell lines (from American Type Culture Collection). Additional Northern blot analysis of RNA from the following did not show any signal: adult human tissues—bladder, heart, kidney, liver, lung, neuroblastoma tumor, skeletal muscle, skin, spleen, and testis; human cell lines—HT29 colon cancer, T43 cystic fibrosis trachea, CFPAC cystic fibrosis pancreatic cancer, Hel erythroleukemia, HepG2 liver cancer, and four melanoma lines.

retina library with AS321 as probe. These clones were characterized by restriction mapping and sequence analysis. As expected, most cDNAs were for the major 1.3-kb transcript. However, DD6, a cDNA clone of 2 kb, contained an extended 3′ untranslated sequence. A purified fragment from this region recognized only the larger transcripts by Northern blot analysis (data not shown), suggesting that these were generated by alternate polyadenylylation. Fig. 3 shows the structure of some of the representative cDNA clones and the sequence for the 1.3-kb cDNA species. The nucleotide sequence reveals a 237-amino-acid-long open reading frame (ORF), preceded by a small ORF in another frame. It is of interest to note that one of the cDNAs (DD10) has an in-frame deletion of 105 amino acids. An imperfect polyadenylylation signal, ATTAAA, is present upstream of the poly(A) in the 3′-untranslated region of both the major 1.3-kb and the larger DD6 cDNA. Translation in rabbit reticulocyte lysate of an *in vitro* synthesized AS321 sense RNA identifies a product of ≈30 kDa, slightly higher than predicted (26 kDa) for the putative polypeptide (data not shown).

The polypeptide sequence derived from AS321 cDNA contains two consensus phosphorylation sites for protein kinase A and five for proline-directed protein kinase (21, 22) in the N-terminal region (Fig. 4*A*). A periodic repeat of leucine residues preceded by a basic DNA binding motif, with strong sequence similarity to the proteins of the jun/fos family (23–25), can be identified in the C-terminal region (Fig. 4*B*). The positions of an alanine spacer and a helix-breaking asparagine within the basic motif are also conserved. Interestingly, a hapted arrangement of five arginine residues in the basic motif overlaps with the leucine zipper. The degree and extent of similarity extends beyond the putative DNA binding domain upon comparison with this region of the transforming oncogene v-*maf* from chicken retrovirus AS42 (26). A region of 97 amino acid residues, including the basic motif and leucine zipper, of the putative AS321 polypeptide shows 75% similarity to the v-*maf* oncogene product (74/97 residues with conservative substitutions) (Fig. 4*C*).

Protein Association and DNA Binding Studies. To ascertain whether the AS321 polypeptide forms heterodimers with c-*jun* or c-*fos* gene products, ^{35}S-labeled *in vitro* translated proteins were incubated alone or in pairwise combination. Proteins were immunoprecipitated with jun or fos antisera and analyzed by SDS/PAGE. When jun and fos products were mixed together, either antibody precipitated a fos–jun complex, but the antibodies were unable to coprecipitate the AS321 gene product from the incubation mixture (data not shown). Preliminary glutaraldehyde-crosslinking experiments (19) with *in vitro* translated AS321 polypeptide suggest the formation of a homomultimer (data not shown). However, these results need further confirmation with purified AS321 protein and its specific antisera.

Gel shift assays were performed to determine whether the AS321 gene product bound to the sequence elements recognized by other DNA binding proteins. End-labeled oligonucleotides containing SP1, AP1/TRE, AP2, AP3, and NF1/CTF sequence elements were incubated with the *in vitro* translated AS321 protein either alone or in combination with jun or fos products for 1 hr. The results (not shown) demonstrate that the AS321 gene product does not bind to any of the sequence elements. The jun product either alone or together with fos showed a band shift with the AP1/TRE oligonucleotide.

Evolutionary Conservation of the AS321 Gene and Its Expression in Vertebrate Retina. Southern blot analysis of genomic DNA from several vertebrates, yeast, and *Drosophila* under low-stringency conditions shows that the gene for AS321 is conserved during evolution (Fig. 5). A signal in the *Drosophila* lane is observed with the mouse homolog of AS321 cDNA as probe (data not shown). Hybridizations with various fragments of AS321 cDNA reveal that conservation is limited to the coding region but not to any particular domain.

The AS321 probe detects homologous transcripts in the retina of baboon, bovine, mouse, rat, and frog (Fig. 6). The sizes of mRNAs in baboon retina closely match those in

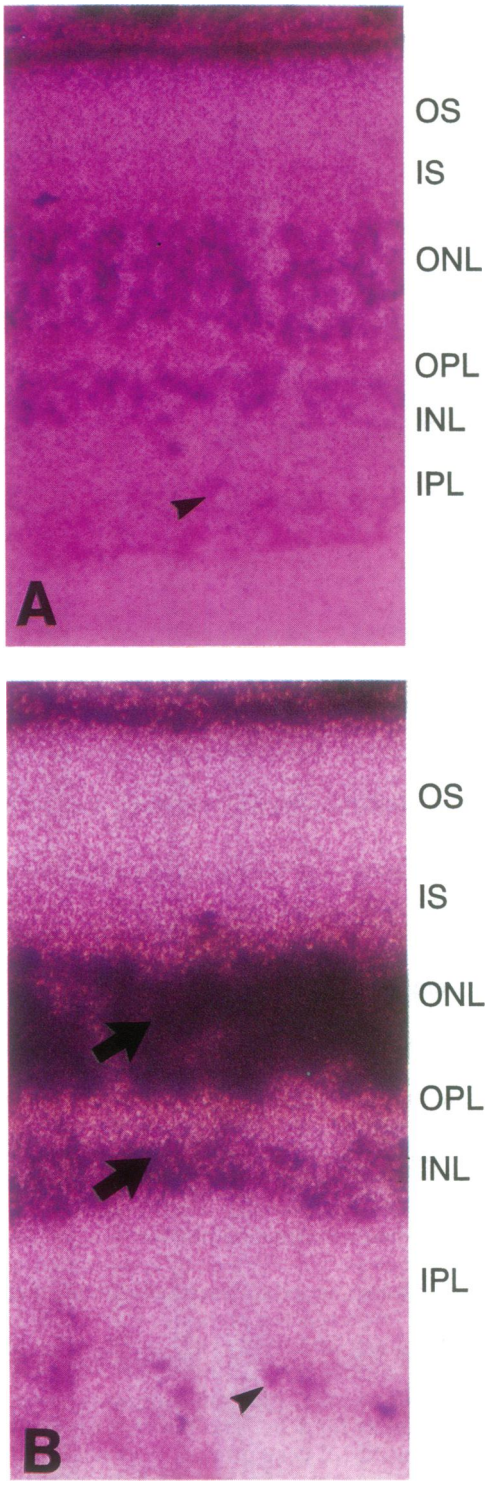
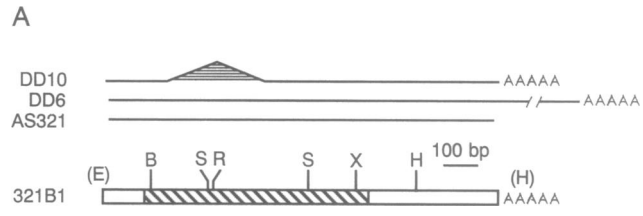


FIG. 2. *In situ* hybridization to RNA in adult baboon retina. ^{35}S-labeled sense or antisense RNA probe for AS321 cDNA was generated *in vitro* by using T7 or T3 RNA polymerase, respectively. Sense (*A*) and antisense (*B*) probes. OS and IS, outer and inner segments of photoreceptors; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. Small arrowheads indicate ganglion cells. Large arrows show specific hybridization.

human, with 1.3 kb being the major species. However, in bovine, mouse, and rat, the major transcript is 2.0 kb. Additional transcripts are observed in bovine and rat retinal RNA. Faint signals at about 2 and 4 kb are detected in the frog RNA. The RNA and cDNA clones from goldfish retina also show weak hybridization with AS321 cDNA, suggesting a



B

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GCTGAGCAGAGGCACCAGGCCCTGCTCCATGGAGCCTTCAGTCTCCTGGGAAGCTGTGCC     60
TGTCTGGCTCTGGCACTGACCACATCCTCTCGGCCATTTCTGAAGTGCACTCCTCCCAGC    120
CCAGCTCCAGAATGGCCCTGCCCCCCAGCCCCCTGGCCATGGAATATGTCAATGACTTTG    180
          M  A  L  P  P  S  P  L  A  M  E  Y  V  N  D  F

ACTTGATGAAGTTTGAGGTAAAGCGGGAACCCTCTGAGGGCCGACCTGGCCCCCCTACAG    240
D  L  M  K  F  E  V  K  R  E  P  S  E  G  R  P  G  P  P  T

CCTCACTGGGCTCCACACCTTACAGCTCAGTGCCTCCTTCACCCACCTTCAGTGAACCAG    300
A  S  L  G  S  T  P  Y  S  S  V  P  P  S  P  T  F  S  E  P

GCATGGTGGGGGCAACCGAGGGCACCCGGCCAGGCCTGGAGGAGCTGTACTGGCTGGCTA    360
G  M  V  G  A  T  E  G  T  R  P  G  L  E  E  L  Y  W  L  A

CCCTGCAGCAGCAGCTGGGGGCTGGGGAGGCATTGGGGCTGAGTCCTGAAGAGGCCATGG    420
T  L  Q  Q  Q  L  G  A  G  E  A  L  G  L  S  P  E  E  A  M

AGCTGCTGCAGGGTCAGGGCCCAGTCCCTGTTGATGGGCCCCATGGCTACTACCCAGGGA    480
E  L  L  Q  G  Q  G  P  V  P  V  D  G  P  H  G  Y  Y  P  G

GCCCAGAGGAGACAGGAGCCCAGCACGTCCAGCTGGCAGAGCGGTTTTCCGACGCGGCGC    540
S  P  E  E  T  G  A  Q  H  V  Q  L  A  E  R  F  S  D  A  A

TGGTCTCGATGTCTGTGCGGGAGCTAAACCGGCAGCTGCGGGGCTGCGGGCGCGACGAGG    600
L  V  S  M  S  V  R  E  L  N  R  Q  L  R  G  C  G  R  D  E

CGCTGCGGCTGAAGCAGAGGCGCCGCACGCTGAAGAACCGCGGCTACGCGCAGGCCTGTC    660
A  L  R  L  K  Q  R  R  R  T  L  K  N  R  G  Y  A  Q  A  C

GCTCCAAGCGGCTGCAGCAGCGGCGCGGGCTGGAGGCCGAGCGCGCCCGCCTGGCCGCCC    720
R  S  K  R  L  Q  Q  R  R  G  L  E  A  E  R  A  R  L  A  A

AGCTGGACGCGCTGCGGGCCGAGGTGGCCCGCCTGGCCCGGGAGCGCGATCTCTACAAGG    780
Q  L  D  A  L  R  A  E  V  A  R  L  A  R  E  R  D  L  Y  K

CTCGCTGTGACCGGCTAACCTCGAGCGGCCCCGGGTCCGGGGACCCCTCCCACCTCTTCC    840
A  R  C  D  R  L  T  S  S  G  P  G  S  G  D  P  S  H  L  F

TCTGAGCCGTTCAGAGCACCTTGTGGTGTAGTGGGGGCTGGGTGGGGTGGCTCCGCCCAG    900
L  *

GAGGCGGCTGCACGGTTCTCTGCATCGTTACCAGAGCGCCTTCTGGTCCTAGCCACGCCC    960
TGTATGACCGCGCAAATATCCCCAAAGCTTTTGGGTCCTCAAGTCATGCCCGAATTTAGA   1020
TGCTGGTCATTTTCTGGAGAGGGGTCCCCTCCCCTTACGAACACAAAAACCCAGCCCACA   1080
TGACTAGCACGCTGAGCTCTGCAGGGACCAGTGCCAGGCACTGGGGGGTGGAAGTGTGGT   1140
GACACAGTGAATGGGAGGTGGAGGAGGGTTGCAGCTCCCACCTCAGTTTAGTTTTTAATT   1200
CAGGGTTTTCAACCTGTAACACATTAAAGCTGTAATTAGCAAAAAAAAAAA            1251
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FIG. 3. (*A*) Structure of cDNA clones. Open and hatched bars show untranslated and coding regions, respectively. 321B1 is the full-length clone for the original AS321 cDNA. DD6 and DD10 are variant clones. B, *Bst*XI; E, *Eco*RI; H, *Hin*dIII; R, *Rsa* I; S, *Stu* I; X, *Xho* I. (E) and (H) are cloning sites for cDNAs. bp, Base pairs. (*B*) Composite cDNA sequence. The complete sequence of AS321 cDNA was obtained in both directions by the dideoxynucleotide chain-termination method. 321B1 extended the sequence of AS321 by 23 and 24 base pairs at the 5′ and 3′ ends, respectively. The single letter code is used for amino acids.

greater sequence divergence between mammals and lower vertebrates (S. Boucher, P. Hitchcock and A.S., unpublished data).

DISCUSSION

Subtraction cDNA cloning is a powerful technique for isolation of tissue-specific genes. We have used an efficient biotin-based method to enrich a human retina library for specific cDNA clones (20). This paper reports a gene that is specifically expressed in all the neuronal cell layers of retina. However, expression is strongest in the photoreceptor cells.

A

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MALPPSPLAMEYVNDFDLMKFEVKREPSEGRPGPPTASLGSTPYSSVPPSPTFSEPGMVGATEGTRPGLEELYWLATLQQ
                                                I

QLGAGEALGLSPEEAMELLQGQGPVPVDGPHGYYPGSPEETGAQHVQLAERFSDAALVSMSVRELNRQLRGCGRDEALRL
                                    II                              e h r

KQRRRTLKNRGYAQACRSKRLQQRRGLEAERARLAAQLDALRAEVARLARERDLYKARCDRLTSSGPGSGDPSHLFL
      basic motif                   leucine zipper
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B

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                |--Basic   motif--||------Leucine      Zipper------|
as321       RLKQRRRTLKNRAYAQACRSKRLQQRRGLEAERARLAAQLDALRAEVARLARERDL--Y
jun-B       RiK..Rk.LrNR..A..CR.rkLe....LE.....L.Ae...L......L-RE-qvaqL
jun-D       RiK..Rk.LrNR..A..CR.rkLe....LE.....L.sQ...L.s..s.L-RE-qvaqL
c-jun       RiK..Rk.mrNR..A..CR.rkLe....LE.....L.AQ...L.s....L-RE-qvaqL
c-fos       ..K.R.R..rNk..A..CR.rR.e....LqAE...L..e..AL..EiA.L.kEke.--L
fos-B       ..K.R.R..rNk..A..CR.rR.e....LqAE...L..e...L.sEiA.L.kEke.--L
fra-1       ..r.R.R..rNk..A..CR.rR.e....LqAE..kL..e..gL..Ei..L.kqke.--L
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C

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        |----------- e h r --------||--Basic motif--||--------Leucine        Zipper---------|
as321   eRFSDaaLVsMSvRELNRQLRGcgrdEalRLKQrRRTLKNRGYAQaCRsKRlQQRrgLEaErarLaaQldaLraEvaRLaRERDlYKarcdrLtSsG
        ||||| || ||||||||||||  ||| ||||||||||||||||| || ||||||| || ||  |  ||| || || || |||| || | ||| | |
AAFVMAF DRFSDEQLVTMSMRELNRQLRGVSKEEVIRLKQKRRTLKNRGYAQSCRFKRVQQRHVLESEKNQLLQQVEHLKQEISRLVRERDAYKEKYEKLVSNG
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FIG. 4. Analysis of AS321 polypeptide. (*A*) Two consensus phosphorylation sites for protein kinase A [K/RK/RX (X)S/T] and five for proline-directed protein kinase (X S/TP X), and the leucines (L) in the zipper are indicated in boldface type. Basic motif and leucine zipper domains are based on similarity with jun/fos oncoproteins. I is a region rich in proline, serine, and threonine residues (P, 9/30; S+T, 10/30). ehr and II represent v-*maf* extended homology region and proline-rich domain (similar to A2 in jun proteins), respectively. The region between I and II has a number of glutamine and glutamic acid residues (Q+E, 12/47). (*B*) Similarity of AS321 polypeptide with proteins of the jun/fos family. (*C*) Similarity between products of AS321 and v-*maf* oncogene (AAFVMAF).

A unique property shared between the photoreceptor and bipolar neurons of neural retina is that the cGMP-mediated signal transduction pathway is turned off in response to the external stimulus—i.e., light for photoreceptors and glutamate for bipolar cells (27, 28). It will be of interest to see if the AS321 gene product has a function in signal transduction.

The mRNA for AS321 is not detected in human fetal eye at 11 weeks but it is expressed in 140-day baboon fetal retina (equivalent to ≈7 months in human gestation). At this stage in baboon retina, only a weak signal is observed for rhodopsin mRNA (data not shown). Interestingly, once the expression of the gene for AS321 is turned on, it remains on as shown by Northern blot analysis of RNA from human retina at various stages.

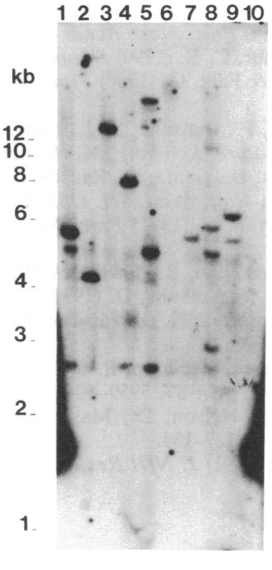

FIG. 5. Zoo blot analysis. Genomic DNA (10 μg) from various organisms was digested with *Hin*dIII for Southern blot analysis. A *Hin*dIII fragment of AS321 cDNA (base pairs 24–989) was used as probe. The conditions for hybridization and washing of Southern blots are as described (17), except that 40% formamide was used. A 1-kb ladder (BRL) is shown on the left. Lanes: 1, human; 2, bovine; 3, dog; 4, pig; 5, cat; 6, rat; 7, mouse; 8, chicken; 9, *Saccharomyces cerevisiae*; 10, *Drosophila melanogaster*.

The basic motif and leucine zipper domains in the AS321 polypeptide have stronger similarity to the proteins of the jun/fos family than to other leucine zipper proteins. Of unusual significance, however, is its homology to the v-*maf* oncogene product. v-*maf* is a transforming oncogene of the AS42 retrovirus, which causes musculoaponeurotic fibrosarcoma in chickens. Molecular analysis of the human AS321 gene shows that an intron interrupts the coding region just upstream of the v-*maf* homologous domain, which is encoded by a single exon (A.J. and A.S., unpublished data). Comparison of the Southern blot analysis of human DNA with AS321 and v-*maf* does not reveal any common bands. The v-*maf* oncogene and its cellular homolog (c-*maf*) probably share only the exon encoding the DNA binding/leucine zipper domain with the AS321 gene. Because of neural retina-specific expression and identification of a leucine zipper in the encoded polypeptide, we propose to name the gene for AS321 *NRL*.

The role of the basic motif in binding to DNA and of the leucine zipper in formation of homo- and heterodimers is well

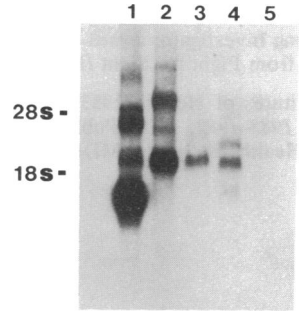

FIG. 6. Expression in vertebrate retina. A Northern blot of total retinal RNA (10–20 μg) was probed with AS321 cDNA (base pairs 24–989) under high stringency (50% formamide; ref. 17). Positions of 28S and 18S RNA are indicated. Lanes: 1, baboon; 2, bovine; 3, mouse; 4, rat; 5, frog (*Rana berlandieri*).

established (23, 24, 29, 30). Dimerization within the family or with other leucine zipper proteins is considered to mediate DNA binding specificity (31). Functional analysis of jun has also defined a proline-rich activation domain upstream of the DNA binding region (32). The position of these domains in the putative NRL polypeptide is similar to those of the jun oncoproteins. However, *in vitro* protein association studies show that the NRL protein does not form heterodimers with the *fos* or *jun* gene products, but it may form homomultimers. The identification of DD10 cDNA encoding a protein with a deletion in the putative activation domain is analogous to the truncated form of FosB that negatively regulates the effects of fos proteins (33). A mutant jun protein lacking the activation domain has been shown to suppress the transformation activity of several oncoproteins (34). Because of the presence of a leucine zipper, the truncated DD10 product may dimerize with the normal NRL polypeptide and influence its biological function. Additional studies are needed to delineate the DNA sequence element recognized by the *NRL* gene product and to identify its counterpart, if any, for heterodimer formation. The presence of consensus phosphorylation sites suggests that the function of the NRL protein may be regulated by phosphorylation.

Recent studies have implicated c-*fos* and related genes in regulation of the circadian clock in response to light signals (35, 36). It will be of interest to see if the expression of *NRL* is affected by the light/dark cycle. The conservation of the *NRL* gene and its expression in retina suggest its involvement in visual function. However, its presence in yeast raises interesting possibilities. It appears that the response to negative growth factors in yeast (α-factor) and neurons (nerve growth factor) leading to cell cycle arrest and differentiation is mediated by common molecular pathways (37). The pattern of *NRL* gene expression during neuronal differentiation in retinal development and its response to external factors should provide insights into unusual signal transducing mechanisms.

We are intrigued by high levels of *NRL* gene expression in mature neural retina, a nonproliferating tissue. Present studies do not permit us to attribute a specific function to its product. Recent evidence from several experimental systems supports the "active" and continuous regulation of differentiation during development (10). Whether *NRL* encodes a cell-type-specific DNA binding protein that acts as a transcriptional regulator of the differentiated neural retinal phenotype and whether its continuous presence is required to maintain retinal function remain to be established.

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