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Expression and Regulation by Interferon of a Double-Stranded-RNA-Specific Adenosine Deaminase from Human Cells: Evidence for Two Forms of the Deaminase

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A 6,474-nucleotide human cDNA clone designated K88, which encodes double-stranded RNA (dsRNA)-specific adenosine deaminase, was isolated in a screen for interferon (IFN)-regulated cDNAs. Northern (RNA) blot analysis revealed that the K88 cDNA hybridized to a single major transcript of ~6.7 kb in human cells which was increased about fivefold by IFN treatment. Polyclonal antisera prepared against K88 cDNA products expressed in *Escherichia coli* as glutathione *S*-transferase (GST) fusion proteins recognized two proteins by Western (immunoblot) analysis. An IFN-induced 150-kDa protein and a constitutively expressed 110-kDa protein whose level was not altered by IFN treatment were detected in human amnion U and neuroblastoma SH-SY5Y cell lines. Only the 150-kDa protein was detected in mouse fibroblasts with antiserum raised against the recombinant human protein; the mouse 150-kDa protein was IFN inducible. Immunofluorescence microscopy and cell fractionation analyses showed that the 110-kDa protein was exclusively nuclear, whereas the 150-kDa protein was present in both the cytoplasm and nucleus of human cells. The amino acid sequence deduced from the K88 cDNA includes three copies of the highly conserved R motif commonly found in dsRNA-binding proteins. Both the 150-kDa and the 110-kDa proteins prepared from human nuclear extracts bound to double-stranded but not to single-stranded RNA affinity columns. Furthermore, *E. coli*-expressed GST-K88 fusion proteins that included the R motif possessed dsRNA-binding activity. Extracts prepared either from K88 cDNA-transfected cells or from IFN-treated cells contained increased dsRNA-specific adenosine deaminase enzyme activity. These results establish that K88 encodes an IFN-inducible dsRNA-specific adenosine deaminase and suggest that at least two forms of dsRNA-specific adenosine deaminase occur in human cells.

Double-stranded RNA (dsRNA)-specific adenosine deaminase (AdD) was first identified as a developmentally regulated dsRNA-unwinding activity in *Xenopus laevis* oocytes (8, 51). dsRNA-specific AdD catalyzes the covalent modification of dsRNA by hydrolytic C-6 deamination of adenosine to yield inosine (9, 69). The resultant A-to-I transitions destabilize the dsRNA helix by disrupting base pairing between the two complementary RNA strands. Hypoxanthine, the base of the nucleotide inosine generated by the RNA editing process, is typically recognized as guanine by the translational and transcriptional machinery (3). dsRNA-specific AdD activity is found primarily in the nucleus and has been demonstrated in a wide variety of animal species (7, 31).

The biological role of dsRNA-specific AdD is not yet well established, although the A-to-I modification has been implicated in two types of functional responses: the establishment of persistent viral infections, and the editing of cellular mRNAs in the brain. dsRNA-specific AdD has been implicated in the host response to viral infection by altering viral RNAs in a manner that may facilitate the development of persistent infections (7, 10). For example, dsRNA-specific AdD is presumed to be responsible for the selective A-to-G and complementary U-to-C biased hypermutation of viral RNAs observed during the replication and subsequent establishment of persistent infection of some negative-stranded RNA viruses, including measles virus (10, 15), parainfluenza virus (46), and vesicular stomatitis virus (49). Most notably, with measles virus, the matrix (M) transcript can become extensively modified with many clustered U-to-C (A-to-G) mutations which prevent subsequent synthesis of functional M protein in patients with subacute sclerosing panencephalitis (SSPE) and measles inclusion body encephalitis (13, 16). The unidirectional biased hypermutations often observed in the M gene also occur, albeit less frequently, in other measles virus genes (6, 17). Measles virus M protein is required for virion assembly and release (20), and when M protein is not expressed, persistent measles virus infection may occur, resulting in a fatal neuropathic response (15).

The dsRNA-specific AdD is also a candidate RNA-editing enzyme capable of catalyzing a selective posttranscriptional modification within the nucleus of specific cellular mRNAs (26, 38). For example, mRNAs in the brain encoding the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and kainate glutamate-gated ion channel GluR receptor subunits are selectively edited, resulting in an altered protein product that affects calcium permeability (26, 34). As first demonstrated for the GluR-B transcript, a specific glutamine CAG codon is converted to a CIG arginine codon at a site designated Q/R; this selective change is dependent upon formation of a dsRNA hairpin structure involving exon 11 and intron 11 sequences near the site of editing (26, 73). In addition, the AMPA receptor subunits GluR-B to -D are also edited in a developmentally regulated manner at a second site, designated R/G, by a conceptually similar mechanism involving exon 13 and intron 13 sequences (38). The AMPA receptor subunit proteins produced from edited RNAs confer increased

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recovery rates from glutamate-mediated desensitization (38). Thus, RNA editing in the brain contributes to the diversity of glutamate receptor proteins that are characterized by an altered electrophysiology.

dsRNA-specific AdD enzymes have been purified to apparent homogeneity and characterized from *Xenopus* oocytes (27), bovine liver (32), and calf thymus (47). The apparent size of these enzymes varies considerably among the three sources, ranging from 83 to 93 kDa for the bovine liver enzyme, 116 kDa for the calf thymus enzyme, and 120 kDa for the *Xenopus* enzyme. However, biochemical and physiochemical analyses indicate that the various forms of the dsRNA-specific AdD proteins all possess the same enzymatic activity. Furthermore, the dsRNA-binding properties reported for dsRNA-specific AdD are similar to those of other known dsRNA-binding proteins, including the interferon (IFN)-induced RNA-dependent protein kinase PKR, which is the prototype of one type of dsRNA-binding motif (22, 27, 41, 64).

As part of our studies on the molecular mechanisms of IFN action (50, 55), we have isolated and characterized cDNA clones of IFN-regulated human proteins, including Mx (1) and PKR (66). K88 was one of the clones that was isolated by a two-stage oligonucleotide-Northern (RNA blot) screening strategy designed originally for the isolation of cDNA clones of IFN-regulated protein kinases (65). However, the K88 cDNA did not encode a protein kinase but rather encoded a novel IFN-induced dsRNA-binding protein present in both the nucleus and cytoplasm of human cells. The molecular cDNA cloning of a human dsRNA-specific AdD was reported independently (33, 48). The cDNA sequence that we obtained for K88 is identical to that reported for the dsRNA-specific AdD from human cells (33). We report here that the K88 cDNA encodes a novel IFN-inducible dsRNA-specific AdD that is present in both the cytoplasm and nucleus of human cells and that this inducible protein of 150 kDa is immunologically related to a 110-kDa protein found in large amounts in the nucleus of both untreated and IFN-treated cells. The identification of an IFN-regulated dsRNA-specific AdD has broad implications for the possible role of RNA editing in the IFN response associated with virus-cell interactions.

MATERIALS AND METHODS

Isolation of K88 dsRNA-binding protein cDNA. The original K88 cDNA clone was obtained by screening a library prepared in λ ZAP (Stratagene Cloning Systems) according to the manufacturer's recommendations, with polyadenylated RNA from IFN-α-treated human amnion U cells (65, 75). Initial screening of the U cell library was done by filter hybridization with a mixture of synthetic 17-mer oligonucleotides corresponding to an amino acid sequence (subdomain VI) which is highly conserved among protein serine/threonine kinases (25). K88-0 was one of several candidate positive plaques which were obtained as part of one of our strategies to molecularly clone protein kinases, including the PKR and cyclic AMP-dependent protein kinase (PKA) kinases (65, 66). K88-0 was subjected to a second round of oligonucleotide screening prior to subcloning into the pBluescript-SK plasmid (Stratagene). Subsequent screening was done by Northern (RNA) blot analysis of RNA isolated from untreated and IFN-treated U cells; this screen revealed that the K88-0 cDNA insert hybridized to an ~6.7-kb mRNA present in IFN-treated cells at greatly elevated levels compared with that in untreated cells. The 550-bp *Eco*RI-*Eco*RI insert from the K88-0 cDNA clone was then sequenced; no homology with known sequences in the GenBank database was found, including those of protein kinases.

The U cell library was then rescreened with the 550-bp fragment from K88-0 as the probe, and several additional clones were isolated, the largest of which was K88-2. Although the K88-0 and K88-2 cDNAs together represented ~2.2 kb of contiguous sequence, no significant open reading frame (ORF) was found. Two commercially obtained libraries, a λ ZAP human placenta library (Stratagene) and a λgt10 random-primed human kidney (Hkr) library (Clontech), were then screened with the 5′ region of clone K88-2 as the probe. Typically, four to five positives were obtained per $\sim 3 \times 10^5$ phage plaques analyzed from these libraries. The largest K88 clones were analyzed by Southern and Northern analysis and then sequenced to confirm overlap with previously isolated clones. This sequential screening strategy was repeated until we obtained a clone (Hkr88-44) which we believed possessed a portion of the 5′ untranslated region (UTR) upstream of a 1,226-amino-acid-encoding ORF (1,226-aa ORF) which we had identified in the K88 clones. Together, the overlapping K88 cDNA clones represented 6,474 nucleotides (nt): a 3′ UTR of 2,749 nt, an ORF of 3,678 nt, and a 5′ UTR of >47 nt (Fig. 1A).

Northern blot analysis. Total U cell RNA isolated by the method of Chirgwin et al. (19) was fractionated by electrophoresis on a 1.5% formaldehyde–agarose gel (10 μg of RNA per lane), blotted to Hybond-N nylon filter membranes, and, after UV cross-linking (Stratalinker 1800), hybridized with the indicated K88 cDNA insert as the ^{32}P-labeled probe. The filter containing RNA isolated from various human tissues was commercially obtained from Clontech. Hybridizations and washings (0.3 × SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% sodium dodecyl sulfate [SDS], 60°C) were carried out essentially as described previously (1, 5).

Southern blot analysis. Phage λ or plasmid DNA was digested with restriction endonucleases, fractionated, and transferred to Hybond-N filter membranes by the method of Southern (63). Filters were probed with K88 cDNA fragments by standard protocols (53).

Sequence analysis. Subclones of the λ K88 cDNA clones were sequenced by the dideoxy chain termination method (58) with the modified T7 DNA polymerase Sequenase according to the manufacturer's instructions (United States Biochemical). Universal primer sites in both single- and double-stranded templates were used, M13mp18/19 (44) and pBluescript SK$^-$ (pBS) (Stratagene), respectively. The dITP protocol was used for GC-rich regions, most notably in the 5′ region of Hkr88-44. Database searches and sequence alignments were performed with the University of Wisconsin Genetics Computer Group software package BLAST, FASTA, and BESTFIT programs on a Silicon Graphics IRIS 4D/340VGX computer.

Construction of K88 dsRNA-binding protein expression vectors. A full-length cDNA clone encoding the human K88 dsRNA-binding protein in the pBluescript vector, designated pBS-K88, was constructed by combining the *Eco*RI (λ)-*Bam*HI fragment (nt 1 to 851) from clone Hkr88-44, the *Bam*HI-*Pst*I fragment (nt 852 to 1836) from clone Hkr88-16, the *Pst*I-*Sal*I fragment (nt 1837 to 2288) from clone Hkr88-2, and the *Sal*I-*Eco*RI (λ linker) fragment (nt 2289 to 3743) from clone Hkr88-15. The following strategy was used to construct pBS-K88. Starting with Hkr88-16 which had been subcloned into the pBS vector at the K88 *Bam*HI (nt 852) and *Eco*RI (λ) (nt 1879) sites, pBS-Hkr88-16 was extended on the 3′ end by inserting the 1.1-kb *Pst*I fragment from Hkr88-2 (K88 nt 1837 to 2974) to generate pBS-Hkr88-16/2 (nt 852 to 2974). The *Bam*HI-*Sal*I fragment (K88 nt 852 to 2288) from pBS-Hkr-16/2 and the *Sal*I-*Eco*RI (λ) fragment (K88 nt 2289 to 3743) from pBS-Hkr88-15 were ligated in a three-way directed reaction into the pBS vector that had been digested with *Bam*HI and *Eco*RI to generate pBS-K88-16/2/15 (nt 852 to 3743), which includes the 3′ portion of the K88 ORF and the K88 TAG termination codon. Next, the *Eco*RI (λ)-*Bam*HI fragment (K88 nt 1 to 851) from pBS-Hkr88-44 and the *Bam*HI-*Hin*dIII(BS) fragment (K88 nt 852 to 3743) from pBS-Hkr88-16/2/15 were ligated in a three-way directed reaction into pBS vector that had been digested with *Eco*RI and *Hin*dIII to generate pBS-K88 (nt 1 to 3743), which includes the entire 1,226-aa ORF of K88, beginning with the ATG initiation codon located at nt 48 to 50 and ending with the TAG termination codon located at nt 3726 to 3728.

The eukaryotic expression vector pcDNA I/Neo (Invitrogen) was used to prepare two different K88 expression constructions, one which encodes the full-length protein K88dsRBP(1–1226) and another which encodes the N-terminally truncated protein K88dsRBP(296–1226), shown in Fig. 8A. Because the 5′ UTR of the K88 cDNA is very GC rich, it was deleted by swapping the *Not*I-*Bgl*II 5′ fragment (nt 1 to 766) from pBS-K88(1–3743) with a *Not*I-*Bgl*II PCR amplification product (52) engineered to delete the 5′ UTR. The Δ5′UTR PCR fragment was synthesized by using the following 5′ primer, designed to include a *Not*I site and the 5′-proximal ATG-specifying M1 of the 1,226-aa ORF encoding the K88 dsRNA-binding protein: 5′-AATGCCTCGCGGCCGCAATGAAT-3′ (K88 nt 31 to 53). The 3′ primer (K88 nt 966 to 947) was 5′-GATAGTCGCA GATTTTCTCC-3′. The Δ5′UTR K88 PCR fragment was digested with *Not*I and *Bgl*II and then subcloned back into the full-length pBS-K88(aa 1–1226) clone to generate pBS-K88(aa 1–1226) Δ5′UTR. The *Not*I(BS)-*Xho*I(BS) fragment from pBS-K88(1–1226) Δ5′UTR was then subcloned into the pcDNA I/Neo vector that had been digested with *Not*I and *Xho*I to generate pcDNA I/Neo-K88(aa 1–1226) Δ5′UTR. The deletion mutant vector pcDNA I/Neo-K88(aa 296–1226), which encodes the N-terminal truncation protein beginning at M296, K88dsRBP(aa 296–1226), was generated by ligation of the *Bam*HI-*Xho*I(BS) fragment (K88 nt 852 to 3743) from pBS-K88(aa 1–1226) into pcDNA I/Neo vector that had been digested with *Bam*HI and *Xho*I.

Production of polyclonal antiserum against recombinant K88 protein. To express recombinant K88 dsRNA-binding protein suitable for antibody production, K88 cDNAs were expressed in *Escherichia coli* BL21(DE3)pLysE as fusion proteins with glutathione *S*-transferase (GST) with the vector pGEX-KG (24). Three different regions of the K88 cDNA were used to prepare rabbit polyclonal antisera against K88 dsRNA-binding protein (Fig. 1B). Antibody 1 (Ab 1) was generated against the region of the K88 cDNA corresponding to aa residues 858 to 1140 with the construct pGST-K88(858–1140). This construction was prepared by inserting the *Eco*RI (λ)-*Hin*dIII fragment from clone Hkr88-8, corresponding to K88 cDNA nt 2619 to 4566, into the *Eco*RI and *Hin*dIII sites of the pGEX vector polylinker. The K88 sequence downstream of the *Kpn*I site (nt 3478),

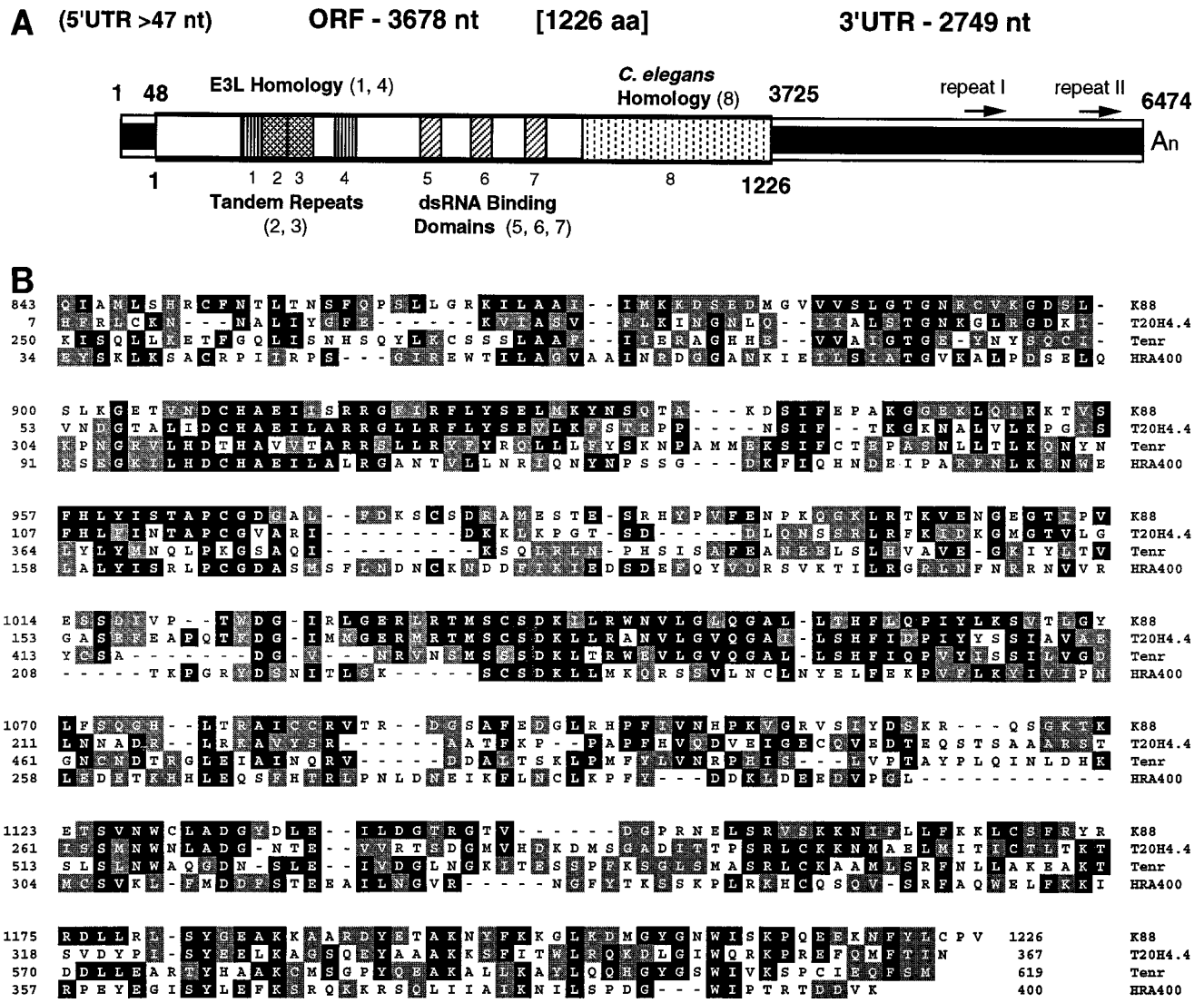

FIG. 1. Molecular cloning of K88 cDNA. (A) Schematic representation of the K88 cDNA encoding dsRNA-specific AdD (accession number U18121). Numbers above the schematic diagram are nucleotide numbers corresponding to the UTRs and the ORF, and numbers below refer to the deduced amino acid sequence. The patterned regions correspond to the homologies indicated, numbered 1 to 8, as follows: 1 and 4, the repeated region of homology with the vaccinia virus E3L protein; 2 and 3, the unique tandem repeat; 5, 6, and 7, the three copies of the dsRNA-binding domain core; and 8, the region of homology with the murine Tenr, *C. elegans* T20H4.4, and *S. cerevisiae* HRA400 gene products. (B) Comparison of the K88 dsRNA-specific AdD C-terminal domain sequence corresponding to the proposed deaminase catalytic domain with the highly conserved sequences of the predicted *C. elegans* T20H4.4 gene product (accession number U00037), the spermatid nuclear RNA-binding Tenr protein from mouse cells (accession number X84693), and the *S. cerevisiae* HRA400 gene product (accession number Z49149). Identical amino acid residues and similar residues are shown as white letters on black and gray backgrounds, respectively.

which includes the 3′ UTR and coding region 3′ to the *Kpn*I site, was deleted by cutting with *Kpn*I and *Hin*dIII, blunting with T4 DNA polymerase, and closing with T4 DNA ligase. Ab 2 was generated against the region of the K88 cDNA corresponding to aa residues 268 to 748 with the construct pGST-K88(268–748). This construction was prepared by inserting the *Bam*HI-*Sal*I fragment from pBS-K88(1–1266) corresponding to K88 cDNA nt 852 to 2288 into the *Bam*HI and *Sal*I sites of the pGEX vector. Ab 3 was generated against the region of the K88 cDNA corresponding to aa residues 9 to 240 with the construct pGST-K88(9–240). This construction was prepared by inserting the *Bam*HI(BS)-*Hin*dIII(BS) fragment from pBS-Hkr88-63(*Eco*RI) corresponding to K88 cDNA nt 70 to 813 into the *Bam*HI and *Hin*dIII sites of the pGEX vector. The 3′ end of the K88 sequence corresponding to nt 769 to 813 was then deleted by cutting with *Bgl*II and *Hin*dIII, blunting with T4 DNA polymerase, and closing with ligase to generate pGST-K88(9–240). The GST-K88 fusion proteins were isolated from the insoluble fraction of freshly transformed *E. coli* by discontinuous Tris-glycine-buffered SDS-polyacrylamide gel electrophoresis (PAGE) (11). Proteins were detected by staining gels with Coomassie blue R-250 dissolved in H_2O; gel slices containing the fusion protein were excised and used for antibody production, which was carried out at Berkeley Antibody Company (Richmond, Calif.). Initial rabbit immunization was done with approximately 500 μg of fusion protein (the protein reference was bovine serum albumin); subsequent boosts with 250 μg of protein were done at 3-week intervals. Blood samples were taken 10 days after each boost.

Western immunoblot analysis. Western immunoblotting was performed by the method of Towbin et al. (67) as previously described (68). Nitrocellulose filter membranes were typically probed with K88 dsRNA-binding protein antiserum at a serum dilution of 1:500 unless otherwise specified; detection of antibody-antigen complexes was done with ^{125}I-labeled protein A (0.05 mCi/ml; ICN) and autoradiography. Unless otherwise noted, SDS-PAGE was carried out with 10% gels.

Cell culture, IFN treatment, and transfection. Human amnion U (57) and neuroblastoma SH-SY5Y (12) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (HyClone) at 5 and 15% (vol/vol), respectively. Monolayer cultures, when about 90% confluent, were treated with 1000 IU of Sendai virus-induced leukocyte IFN-α (generously provided by K. Cantell, Helsinki, Finland) per ml or recombinant IFN-

αA/D (generously provided by S. Pestka, Piscataway, N.J.) per ml for 16 h unless otherwise specified. Parallel cultures were left untreated as controls.

Monkey kidney COS-1 cells (21) were grown in DMEM supplemented with 5% fetal bovine serum. pcDNA I/Neo eukaryotic cell expression vector constructions were introduced into COS cells in monolayer culture by the DEAE-dextran-chloroquine phosphate transfection method with 5 μg of DNA per ml (39, 45).

Indirect immunofluorescence microscopy. Human U cells were seeded onto poly-L-lysine-treated glass coverslips (18 mm) in eight-well microtiter dishes and either IFN treated or left untreated, after which they were prepared for immunofluorescence microscopy essentially as described by Jordan et al. (30). Briefly, cells were fixed for 10 min at room temperature in phosphate-buffered saline (PBS) containing 10% formalin and 2 mM EGTA (ethylene glycol tetraacetic acid), drained, and then transferred to 100% methanol containing 2 mM EGTA for 10 min at −20°C. After washing with PBS–0.1% bovine serum albumin (BSA), the samples were blocked (20 min, room temperature) with normal goat serum (1:4 dilution in PBS) before incubation (1 h, 37°C) in a moist chamber with either the specified K88 immune serum or preimmune serum (1:333 dilution in PBS). The cells were then washed four times for 5 min each with PBS-BSA before incubation with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (IgG; Cappel, Inc.) at 37°C for 1 h, drained, and stained with DAPI (4,6-diamino-2-phenylindole; Sigma) at a concentration of 1 μg/ml for 1 min. Cells were rinsed and mounted on glass slides with FITC-Guard (Testog, Inc.). Photomicrographs were made with a Zeiss PM III photomicroscope and Kodak T-max 400 film. The film was exposed for the same length of time for both untreated and IFN-treated cells stained with either immune or preimmune serum.

RNA-binding activity. The RNA-binding activity of proteins encoded by the K88 cDNA was measured by two methods, Northwestern (RNA-protein) blot analysis and RNA-Sepharose affinity chromatography. Northwestern analysis was carried out as previously described (41). Briefly, GST-K88 fusion proteins expressed in *E. coli* were fractionated by SDS-PAGE and transferred to nitrocellulose filter membranes, which were then probed with ^{32}P-labeled human immunodeficiency virus (HIV) TAR RNA as the probe. Nitrocellulose filter blots prepared in parallel were subjected to Western analysis with anti-K88 antibody.

RNA-Sepharose affinity chromatography was carried out essentially as described by Lasky et al. (36). Briefly, extracts prepared from IFN-treated U cells by Nonidet P-40 (NP-40) lysis were used to isolate nuclei by centrifugation at 10,000 × *g* for 5 min (59). The crude nuclear pellet (from a 150-mm monolayer culture) was suspended in 200 μl of 30 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer, pH 7.5, containing 0.6 M KCl, 5 mM magnesium acetate, and 1 mM dithiothreitol (DTT) and then sonicated. The S100 supernatant solution obtained by centrifugation at 100,000 × *g* for 1 h in a Beckman TL100 ultracentrifuge was dialyzed against 100 volumes of column-binding (CB) buffer (30 mM HEPES buffer [pH 7.5] containing 0.12 M KCl, 3 mM magnesium acetate, 1 mM DTT, and 10% [vol/vol] glycerol), which was changed once after 6 h. The dialysate was centrifuged at 100,000 × *g* for 1 h to remove any remaining particulate material prior to affinity chromatography. RNA-Sepharose columns (0.1 ml), either double-stranded poly(rI):poly(rC) or single-stranded poly(rA), were prepared in 1-ml syringes by washing with several volumes of CB buffer containing BSA (10 mg/ml) followed by 20 volumes of CB buffer alone. Nuclear extract (100 μl) was applied to the RNA-Sepharose column, and the flowthrough fraction was recycled over each column three times. The columns were then washed with 20 volumes of CB buffer; proteins bound to the RNA-Sepharose matrix were eluted with 100 μl of SDS gel-loading buffer by heating for 5 min at 100°C. The supernatant solution obtained by microcentrifugation was analyzed by SDS-PAGE and Western blotting.

dsRNA-specific AdD assay. dsRNA-specific AdD assays were carried out essentially as described by O'Connell and Keller (47). Crude nuclear extracts were prepared, from either untreated or IFN-treated SH-SY5Y cells or K88 cDNA vector-transfected COS cells, by the NP-40 lysis procedure as described for measurement of RNA-binding activity by affinity chromatography. Synthetic dsRNA substrate was prepared by transcription in vitro with the pBS vector containing an AT-rich 512-nt *Hin*dIII fragment from a PKR genomic subclone (generously provided by K. Kuhen of this laboratory). Opposing transcripts were generated by linearizing the plasmid with either *Eco*RI or *Xho*I and then transcribing with either T7 or T3 RNA polymerase (Promega), respectively. Transcripts were annealed by heating to 75°C for 5 min and then slowly cooling to room temperature. The RNA strand generated with T7 polymerase was ^{32}P labeled with [α-^{32}P]ATP (3,000 Ci/mmol; Amersham). The standard reaction mixture (25 μl) for measurement of AdD activity included 5 fmol of ^{32}P-labeled dsRNA and nuclear extract protein as indicated in 50 mM Tris-HCl buffer (pH 7.9) containing 50 mM KCl, 5 mM EDTA, 10% (vol/vol) glycerol, 1 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). After incubation at 30°C for 2 h, the dsRNA substrate was recovered by ethanol precipitation with 0.5 μg of poly(rI) added as carrier RNA. The precipitated RNA was washed with 70% ethanol, dried, and suspended in 10 μl of nuclease P1 buffer (30 mM potassium acetate [pH 5.3], 10 mM $ZnSO_4$) before digestion with 1.5 μg of nuclease P1 (Pharmacia) for 1 h at 50°C (47). IMP, AMP, ATP, and dsRNA were resolved from each other by thin-layer chromatography (TLC) on cellulose NM 300 glass plates (Macherey and Nagel) in a solvent consisting of saturated $(NH_4)_2SO_4$, 100 mM sodium acetate (pH 6.0), and isopropanol (79:19:2). Autoradiography was usually done for 16 h at −80°C with a screen, and quantitation of excised TLC spots was done with a Beckman LS1801 liquid scintillation system.

Nucleotide sequence accession number. The GenBank accession number of the human K88 cDNA sequence is U18121.

RESULTS

Molecular cloning and sequence analysis of K88 cDNA. Hybridization with mixed oligonucleotide probes corresponding to conserved catalytic subdomains of protein kinases had been shown to be a viable approach to the isolation of previously uncharacterized kinases (25). By coupling oligonucleotide probing with a subsequent Northern hybridization screen with RNA isolated from cells under various conditions of IFN treatment (65), a set of cDNA clones encoding candidate protein serine-threonine kinases regulated by IFN was isolated, such as PKR and a splice site variant of PKA (56, 65, 66). Also among the six IFN-regulated cDNA clones isolated was KIN88-0. Because the *Eco*RI insert from KIN88-0 hybridized to a single large transcript that was much more abundant in IFN-treated than in untreated human cells, a complete cDNA (designated K88) was isolated by sequential screening of cDNA libraries (see Materials and Methods).

The consensus sequence for the K88 cDNA was determined by sequence analysis of representative independently isolated cDNA clones. A schematic representation of the K88 cDNA is shown in Fig. 1A. The 6,474-nt cDNA possessed a single long ORF, beginning with an ATG codon at nt 48 to 50 and terminating with a UAG codon at nt 3726 to 3728. The deduced K88-encoded protein consisted of 1,226 aa, with a calculated molecular mass of 136 kDa. The −3/+4 flanking nt of ATG (nt 48) were purines, characteristic of an optimal sequence for initiation of translation in vertebrates (35). The sequence upstream of ATG (nt 48) was GC rich (82% GC) and lacked additional in-frame ATG codons, indicating that the K88 cDNA likely contained the complete protein-coding sequence and part of the 5′ UTR. The 3′ UTR was unusually long, 2,749 nt, and included the AATAAA polyadenylation signal (71) found 17 nt upstream of a poly(A) tail. In addition, the 3′ UTR included a region of 227 nt of unknown function that was repeated with 87% identity (Fig. 1A), as well as three copies of the ATTTA sequence associated with mRNA instability (14). The deduced amino acid sequence from the K88 cDNA is identical to that reported by O'Connell et al. for the human dsRNA-specific AdD (48). Our K88 cDNA sequence for the human deaminase differed from that reported by Kim et al. (33) at a single position in the coding region: a G instead of an A at nt 1198, resulting in the substitution of an arginine for lysine at position 384. We also observed one change in the 5′ UTR, a G instead of an A at nt 29, and changes at five positions in the 3′ UTR: a T instead of a C at nt 4348 and also at nt 4586; a G instead of an A at nt 5519; an additional G after nt 5671; and an 8-nt insertion (GCCCCCAT) after position 6046.

The 1,226-residue sequence deduced from the consensus sequence for the K88 cDNA possessed regions of homology with several known dsRNA-binding proteins, with the vaccinia virus protein E3L (2), and with the deduced products of the *Caenorhabditis elegans* T20H4.4 (72), murine Tenr (60), and *Saccharomyces cerevisiae* HRA400 genes. Three copies of the dsRNA-binding subdomain R motif (22, 41, 64) were found in K88 (Fig. 1A). A comparison of the K88 R motifs with each other and with those from other mammalian proteins showed striking conservation of the R motif core residues. The core residues established by mutagenesis of the prototype dsRNA-dependent protein kinase PKR to be important for dsRNA-

binding activity were conserved (23, 41–43). The N-terminal region of the K88 deduced sequence also included a 26-residue region that was repeated at aa positions 170 to 195 and 328 to 353 (35% identity, 68% similarity) and displayed significant homology with the N-terminal region of the vaccinia virus E3L protein (31% identity, 58% similarity) (2). This region of homology between K88 and E3L was in addition to the homology corresponding to the dsRNA-binding R motif (41). The E3L-like repeats within K88 were interspersed with a separate set of tandem repeats showing 79% identity (86% similarity) with each other over 49 aa, but with no significant homology to sequences represented in the data bank (Fig. 1A). The C terminus of K88 included a 380-aa region (Fig. 1B) that has extensive homology with the entire predicted *C. elegans* T20H4.4 gene product (37% identity, 63% similarity), the *S. cerevisiae* HRA400 gene product (23% identity, 49% similarity), and the C terminus of the murine Tenr protein (29% identity, 51% similarity), three proteins of unknown function (60, 72). The sequence obtained for the human K88 cDNA showed near identity with the sequences reported for the dsRNA-specific AdDs from human (33, 48) and rat (48) cells.

K88 cDNA probes hybridize to an IFN-inducible 6.7-kb mRNA. Northern blot analysis showed that the K88 cDNA hybridized to a single 6.7-kb mRNA (Fig. 2). This mRNA was more abundantly expressed in human amnion U cells treated with IFN than in untreated cells and, at high stringency of hybridization and washing, was not detected in either untreated or IFN-treated mouse fibroblast L cells (Fig. 2A). The IFN induction kinetics of K88 mRNA expression in U cells treated with a saturating concentration of IFN-α for various periods of time were examined by Northern analysis. As shown in Fig. 2B, the maximum level of K88 transcript was observed after 6 h of IFN treatment. The increase in the amount of the 6.7-kb transcript was about fivefold after 6 h of IFN treatment, following a lag of at least 2 h during which no substantial change in transcript level was detected. Comparable amounts of RNA from untreated and IFN-treated cells were present on the Northern gel blots, as measured by levels of 18S and 28S rRNA (data not shown) and the level of KIN30 transcript present (Fig. 2B), an RNA not known to be regulated by IFN (66). The pattern of K88 mRNA expression in various human organs was also determined. In agreement with others (33, 48), a major RNA species of 6.7 kb was detected in all tissues examined, including heart, brain, lung, liver, skeletal muscle, kidney, and pancreas (data not shown).

The 6.7-kb RNA was the only mRNA detected in cell lines and various organs when hybridization probes that corresponded to different regions of the K88 cDNA were used, including a probe (*Eco*RI-*Kpn*I) from the highly conserved C-terminal region of the ORF, a probe [*Eco*RI (λ)-*Bam*HI, nt 1 to 851] that included the 5′ UTR, and a probe (*Kpn*I-*Eco*RI) from the 3′ UTR (Fig. 2 and data not shown). Furthermore, from the apparent size of the single mRNA detected in human cell lines and organs, about 6.7 kb (Fig. 2), we concluded that our 6.5-kb K88 cDNA (Fig. 1A) represented a nearly full-length copy of the K88 mRNA.

K88-specific immune sera recognize two proteins in human cells, an IFN-inducible 150-kDa protein and a constitutively expressed 110-kDa protein. Rabbit immune sera prepared against three different regions of the K88 cDNA product expressed in *E. coli* as GST-K88 fusion proteins were used to analyze protein expression in untreated and IFN-treated human cell lines by Western immunoblot analysis (Fig. 3A). Two proteins were detected, both in human neuroblastoma SH-SY5Y cells (Fig. 3B and C) and in human amnion U cells (Fig. 4A), by the K88-specific immune sera. One protein was a

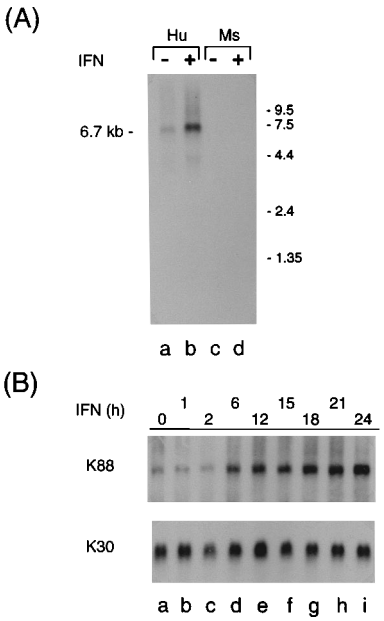

FIG. 2. IFN-inducible expression of K88 transcripts. (A) Induction by IFN of a ~6.7-kb RNA transcript; Northern blot analysis of total RNA purified from human amnion U cells (Hu) and murine L cells (Ms). Cells were either left untreated (−) or treated with IFN (+). The filter was hybridized with the *Eco*RI-*Kpn*I K88 ORF fragment (nt 2277 to 3470) (see Fig. 3A) from pBS-K88. The positions of RNA size markers are given in kilobases. The position of the ~6.7-kb K88 transcript is shown. (B) Kinetics of IFN induction of K88 transcripts; Northern blot analysis of total RNA isolated from human U cells treated with IFN for the specified period of time (in hours). The filter was hybridized to the K88 probe as described for panel A. As a control for RNA loading and integrity, the same filter was rehybridized to the KIN30 probe. KIN30 is not regulated by IFN (66).

150-kDa IFN-inducible protein, and the other was a constitutively expressed 110-kDa protein. K88 Ab 3, directed against a 232-aa N-terminal portion of K88, was monospecific and recognized only the IFN-inducible p150 protein in human SH-SY5Y cells (Fig. 3B, lanes k and l) and human U cells (Fig. 4A). By contrast, K88 Ab 2 and K88 Ab 1 recognized both proteins, the IFN-inducible p150 and the constitutive p110, in human cell lines. K88 Ab 1 was directed against a 283-aa C-terminal portion of K88 corresponding to the region with homology to the mouse Tenr, *C. elegans* T20H4.4, and *S. cerevisiae* HRA400 proteins, and K88 Ab 2 was directed against a 481-aa central portion of K88 that included two of the dsRNA-binding R motifs. Neither the IFN-inducible p150 protein nor the constitutively expressed p110 protein was recognized by preimmune serum (Fig. 3B and 4A).

Fractionation of cell extracts into nuclear and cytoplasmic fractions revealed that the constitutively expressed p110 protein was primarily nuclear, whereas the IFN-inducible p150 protein was found in comparable amounts in both the nuclear and cytoplasmic fractions in human SH-SY5Y (Fig. 3B) and U (Fig. 4A) cells. The cytoplasmic p150 was pelleted by centrifugation at 100,000 × *g* for 1 h and could be partially solubilized from the P100 pellet by treatment with buffers containing high salt concentrations (>0.5 M KCl) but not nuclease. When the distribution of the IFN-inducible dsRNA-dependent protein kinase PKR was examined as an internal control with the same samples, PKR predominantly localized to the cytoplasmic fraction, consistent with previously published observations (29, 54, 66). Although both PKR and the K88 predicted proteins contain multiple copies of the conserved R motif involved in

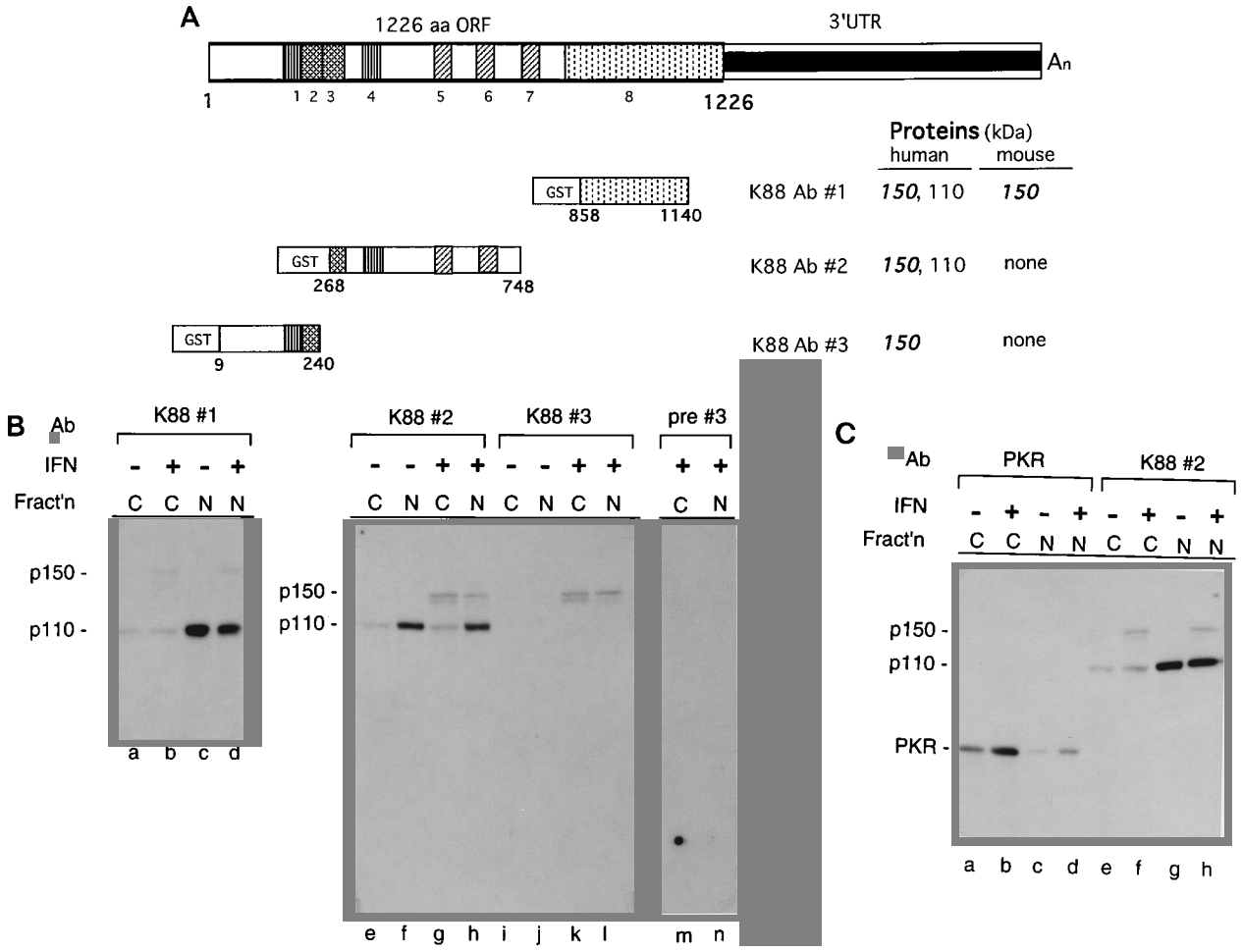

FIG. 3. K88 protein expression. (A) Schematic representation of the K88 cDNA regions expressed as GST-K88 fusion proteins and used for production of K88-specific antibodies. The three GST-K88 fusion proteins used to immunize rabbits for generation of immune sera (designated Ab 1, 2, and 3) are indicated. The amino acid residues corresponding to the deduced K88 cDNA ORF are shown below each construction. (B) Recognition of an IFN-inducible 150-kDa protein (p150) and a constitutively expressed 110-kDa protein (p110) in human neuroblastoma SH-SY5Y cells. Western immunoblot analysis of K88 dsRNA-specific AdD protein expression in cytoplasmic (C) and crude nuclear (N) fractions prepared from untreated (−) and IFN-treated (+) SH-SY5Y cells. Analysis was done with the three different K88-specific immune sera (Ab 1, Ab 2, and Ab 3) and preimmune serum (pre #3), as indicated. (C) As controls, blots parallel to those analyzed in panel B were probed either with K88 immune serum from rabbit 2 (K88 2) or with RNA-dependent protein kinase-specific immune serum (PKR).

dsRNA binding, antisera directed against the two proteins did not cross-react; K88 Ab 2 did not recognize PKR (Fig. 3B and C), and anti-PKR did not recognize either the p150 or p110 protein (Fig. 3C).

Even though the IFN-inducible p150 protein was recognized by all three K88-specific antisera in human cell lines, somewhat surprisingly, only the anti-human K88 Ab 1 serum was cross-reactive against mouse L fibroblast cells (Fig. 4B). Furthermore, the K88 Ab 1 immune serum recognized only a 150-kDa protein in mouse cells; a p110-like protein was not detected in L cells. The mouse p150-like protein was IFN inducible and largely cytoplasmic (Fig. 4B).

Quantitation by laser densitometry and by analysis of serial dilutions of total extracts prepared from IFN-treated cells and cells left untreated revealed that p150 expression was increased approximately 5-fold in human U cells, about 10-fold in human SH-SY5Y cells, and at least 10-fold in mouse L cells by IFN treatment. The Western signal observed with a mobility just below that of the p150 signal in some samples likely represented proteolytic degradation of p150 (32). The apparent degradation of p150 varied appreciably between samples, from almost no degradation (Fig. 3B) to as much as 30% (Fig. 4A).

Subcellular localization by immunofluorescence microscopy of IFN-inducible p150 and constitutively expressed p110 proteins. Analysis of crude cytoplasmic and nuclear fractions by Western immunoblotting revealed that the p150 protein was associated with both the nuclear and cytoplasmic fractions, whereas the p110 protein was primarily nuclear (Fig. 3 and 4). Indirect immunofluorescence microscopy of human U cells confirmed that the constitutively expressed p110 protein was restricted to the nucleus, whereas the IFN-inducible p150 protein was present in both the cytoplasm and nucleus (Fig. 5).

Immunostaining of untreated U cells with K88 Ab 2 showed an intense nuclear fluorescence that was somewhat punctate (Fig. 5B). Furthermore, with both U and SH-SY5Y cells, an intense nucleolar staining was also observed with K88 Ab 2 in some but not all cells. Because p110 was the only protein readily detected by Western blotting of untreated U and SH-SY5Y cells with either K88 Ab 1 or Ab 2 (Fig. 3B and 4A), these antisera are effectively immunospecific for p110 in untreated cells.

K88 Ab 3 is monospecific for p150, as ascertained by Western blotting of extracts from IFN-treated cells (Fig. 3 and 4). Little significant immunostaining of untreated U cells was ob-

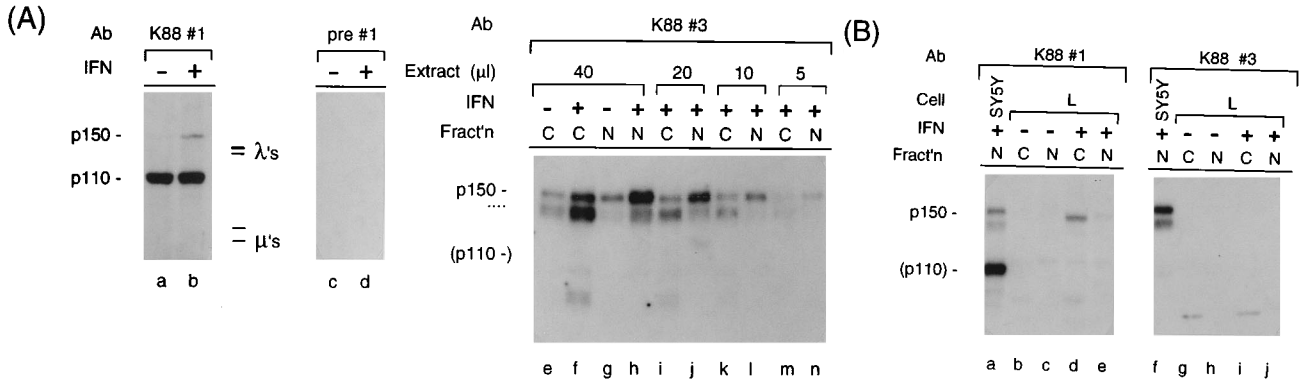

FIG. 4. Expression of the 150-kDa IFN-inducible protein in human and mouse cells. (A) Western immunoblot analysis of K88 dsRNA-specific AdD protein expression in crude extracts prepared from human amnion U cells, either untreated (−) or IFN treated (+). Analysis was done with two different K88-specific immune sera, Ab 1 and Ab 3, or with preimmune serum from rabbit 1 (pre #1). For purposes of quantitation, serial dilutions of U cell cytoplasmic (C) and nuclear (N) fractions were analyzed on an SDS–6% PAGE gel. ^{35}S-labeled reovirus proteins were analyzed in parallel as size standards (λ, 144, 142, and 137 kDa; μ, 83 and 76 kDa). The dashed line marks the presumed proteolytic degradation products of p150. The expected position of p110, which is not recognized by Ab 3 (see Fig. 3B also), is shown in parentheses. (B) Western immunoblot analysis of K88 dsRNA-specific AdD protein expression in cytoplasmic (C) and nuclear (N) extracts prepared from mouse fibroblast L cells, either untreated (−) or IFN treated (+). Analysis was done with two different K88-specific immune sera at a dilution of 1:50, and as a control for the antibody reactivity and to establish the gel mobility positions of p150 and p110, nuclear extract from human SH-SY5Y cells was also analyzed.

tained with K88 Ab 3 (Fig. 5C), but IFN-treated U cells showed a diffuse cytoplasmic staining and, in addition, nuclear staining (Fig. 5D). No immunostaining of IFN-treated U cells was obtained with preimmune serum (Fig. 5A). The DAPI staining pattern (Fig. 5a to d) verified that comparable numbers of cells were present in the fields analyzed (Fig. 5A to D) with the various K88-specific sera. Neither p110 nor p150 appeared to be associated with any uniquely definable structure during mitosis, as judged from the chromosome staining pattern with DAPI compared with the K88 Ab 2 and 3 staining patterns (data not shown).

p150 and p110 are both dsRNA-binding proteins. The 1,226-aa sequence deduced for the K88 cDNA-encoded protein contained three copies of the prototype dsRNA-binding R motif (Fig. 1A). Therefore, K88 represented a novel IFN-induced protein that potentially possessed dsRNA-binding activity. Because we had successfully used a Northwestern blot assay with TAR RNA as the probe to identify the dsRNA-

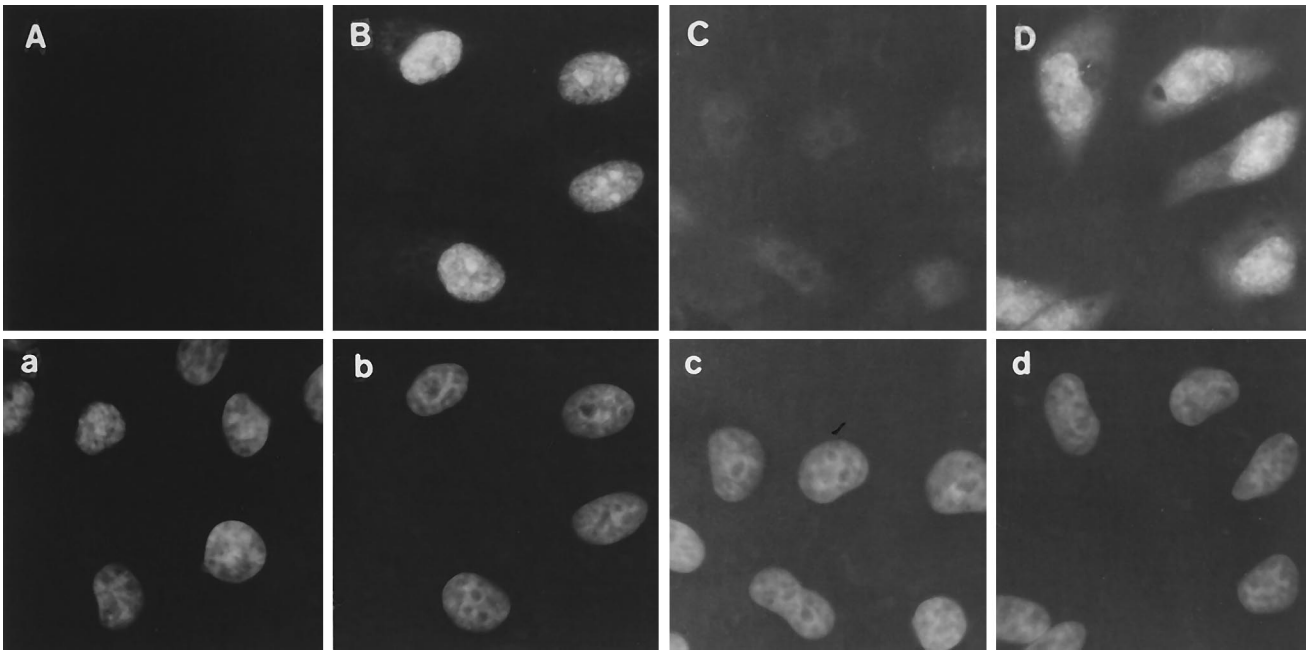

FIG. 5. Subcellular localization of p150 and p110 proteins in human cells by immunofluorescence microscopy. Expression of the IFN-inducible 150-kDa protein (p150) and the constitutively expressed 110-kDa protein (p110) in human amnion U cells was examined by indirect immunofluorescence microscopy. Cells, either untreated or IFN treated as specified, were fixed and then stained with the indicated rabbit serum before counterstaining with FITC-conjugated goat anti-rabbit IgG serum. (A) IFN-treated cells stained with rabbit preimmune serum; (B) untreated cells stained with Ab 2 immune serum, which is selective for p110 in untreated cells; (C and D) untreated (C) and IFN-treated (D) cells stained with Ab 3 immune serum, which is specific for p150. (a to d) Chromosome staining pattern obtained with DAPI of the same fields of cells as shown in panels A to D, respectively.

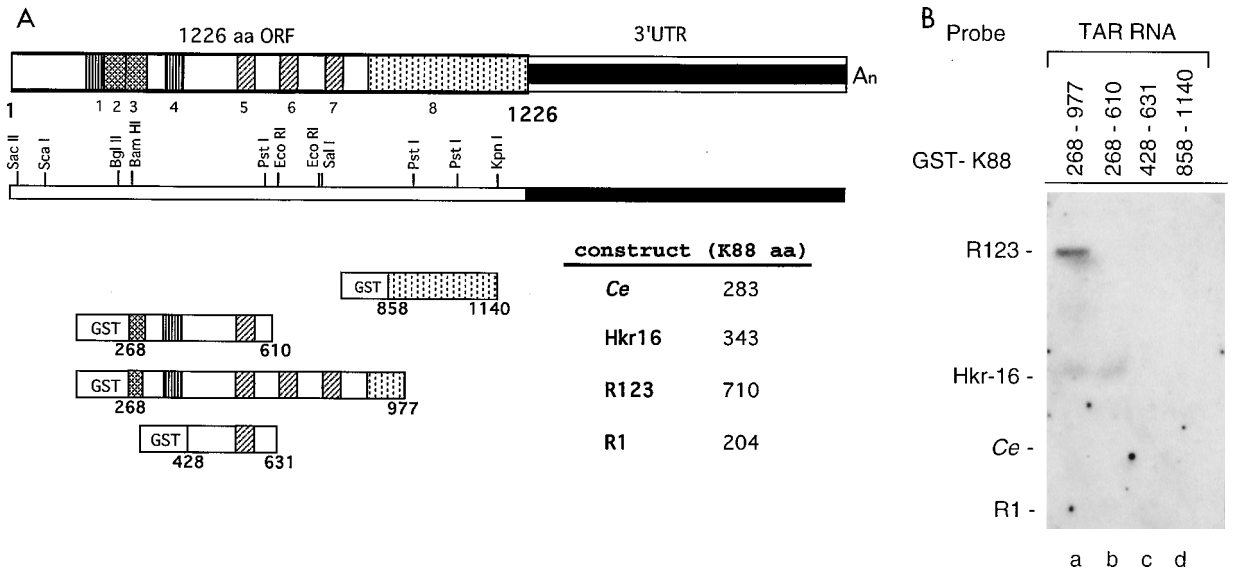

FIG. 6. RNA-binding activity of recombinant GST-K88 fusion proteins synthesized in *E. coli*. (A) Schematic representation of the pGEX-K88 expression constructions used to produce GST-K88 fusion proteins. The three dsRNA-binding subdomain R motifs are denoted by the three hatched boxes. The GST-K88 fusion from the C-terminal region designated Ce did not contain any R motifs; the Hkr16 and R1 fusions each contained one copy and the R123 fusion contained three copies of R. The amino acid residues corresponding to the deduced K88 cDNA ORF are shown below each construction. (B) Northwestern RNA-binding analysis of GST-K88 fusion proteins performed with ^{32}P-labeled human immunodeficiency virus TAR RNA. Proteins were fractionated by SDS-PAGE (7.5% gel) and transferred to nitrocellulose filter membranes. Approximately 20 pmol of each GST-K88 fusion protein was analyzed.

binding R motif of PKR (41, 42), we initially used this approach to examine K88 cDNA-encoded products for dsRNA-binding activity. GST-K88 fusion proteins were produced in *E. coli* (Fig. 6A) and examined for dsRNA-binding activity. The R123 GST-K88 fusion, which possessed all three copies of the R motif, bound both TAR RNA (Fig. 6B) and VA_IRNA (data not shown) much better than either the Hkr16 or R1 GST-K88 fusions, which possessed only one copy of the R motif. The Ce GST-K88 fusion generated from the C-terminal region of K88 lacked R motifs, and it did not bind either TAR RNA (Fig. 6B) or adenovirus VA_IRNA (data not shown). Likewise, the GST portion of the fusion protein expressed from the vector alone did not bind RNA. As a control, Western analysis with K88-specific antibody verified that the Ce, Hkr16, and R1 fusion proteins were present on the blot even though no Northwestern signal was obtained with them (data not shown).

To assess the p150 and p110 proteins synthesized in human cells for their dsRNA-binding activity, nuclear extracts were prepared from IFN-treated U cells and analyzed by RNA-Sepharose affinity chromotography and Western blotting (Fig. 7). Both p150 and p110 bound to the dsRNA affinity column, poly(rI) · poly(rC)-Sepharose (Fig. 7, lane d). However, neither p150 nor p110 bound to the single-stranded RNA affinity column, poly(A)-Sepharose (Fig. 7, lane f). As a control, Western analysis of the same affinity column samples with anti-PKR immune serum demonstrated that the RNA-binding characteristics of the K88-specific p150 and p110 proteins (Fig. 7) were similar to those of the well-characterized PKR protein (56).

K88 encodes an IFN-inducible dsRNA-specific AdD. The sequence that we had obtained for the IFN-inducible K88 cDNA showed near identity with the sequences for dsRNA-specific adenosine deaminase from human and rat cells reported by Kim et al. (33) and O'Connell et al. (48). To verify that the K88 cDNA encoded an AdD enzyme, the K88 cDNA under the control of the cytomegalovirus promoter was expressed in transfected COS cells, and extracts were analyzed for AdD activity (Fig. 8). Because of the high GC content (>80%) of the 5′ UTR and adjacent 5′ portion of the ORF, two approaches were taken to express the K88 cDNA in transfected cells (Fig. 8A). The vector pcDNA I/Neo-K88(aa1–1226) Δ5′UTR was engineered to express the full-length protein, K88dsRBP(1–1226), in the absence of the 5′ UTR but from the authentic translation start site, M1. The deletion mutant pcDNA I/Neo-K88(aa 296–1226) was engineered to also lack the GC-rich portion of the ORF and used M296 as the translation start site. The full-length K88 dsRNA-binding protein was poorly expressed, whereas the truncated product K88dsRBP(296–1226) was efficiently expressed in COS cells, as measured by Western blotting (Fig. 8B). Nuclear

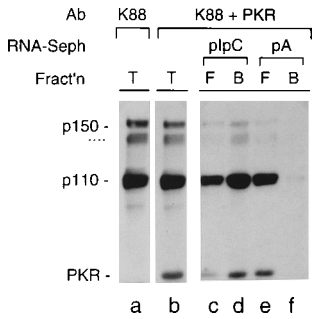

FIG. 7. RNA-binding activity of p150 and p110 proteins synthesized in human cells. RNA-Sepharose affinity chromatography was performed with nuclear extracts prepared from IFN-treated human U cells. Extracts were analyzed with either poly(rI) · poly(rC) (pIpC)-Sepharose or poly(A) (pA)-Sepharose. The flowthrough (F) and column-bound (B) fractions from each column, as well as starting material (T), were fractionated in parallel by SDS-PAGE (6% gel) and transferred to nitrocellulose for Western analysis. The filter was first probed with a mixture of K88-specific Ab 2 and 3 to enhance the signal for p150 (lanes a to f) and then reprobed with PKR immune serum (KIN88+PKR) (lanes b to f) as a control for dsRNA-binding activity measured for PKR. The dotted line denotes likely proteolytic fragments from p150.

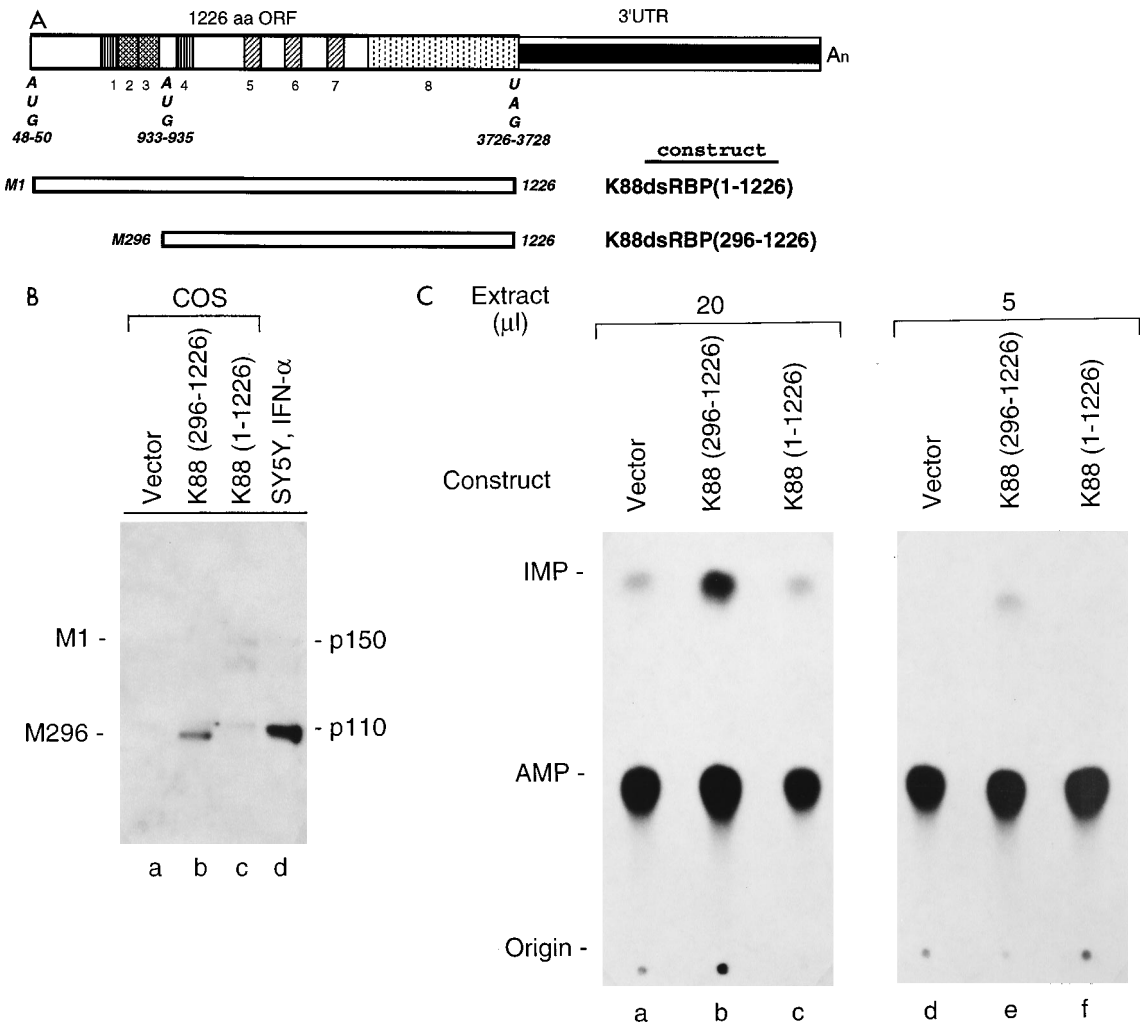

FIG. 8. Expression of K88 cDNA in transfected COS cells. (A) Schematic representation of the K88 animal cell expression constructs. The K88 cDNA is under the control of the cytomegalovirus promoter in the vector pcDNA I/Neo. The authentic AUG initiation codon (nt 48 to 50) was used to express the 1,226-aa full-length K88 cDNA ORF product, designated K88dsRBP(1–1226); the 931-aa N-terminal truncation, designated K88dsRBP(296–1226), was expressed from the internal AUG codon (nt 933 to 935). The authentic UAG stop codon (nt 3726 to 3728) was used for both constructs. (B) Western immunoblot analysis of crude nuclear extracts prepared from monkey COS cells transfected with either the full-length K88 cDNA or the N-terminal truncation of the K88 cDNA in the vector pcDNA I/Neo. Crude nuclear extracts from IFN-treated SH-SY5Y cells were also analyzed to provide a reference standard for the p150 and p110 proteins. Cells transfected with pcDNA I/Neo vector alone were analyzed as a negative control. Extracts were fractionated by SDS-PAGE (6% gel), transferred to nitrocellulose, and probed with a mixture of K88-specific antibodies 2 and 3. (C) dsRNA-specific AdD enzyme activity of extracts prepared from K88-transfected COS cells. ^{32}P-labeled dsRNA substrate was incubated with 20 or 5 μl of crude nuclear extract (0.2 μg/μl) prepared from transfected cells. Products of subsequent P1 nuclease digestion were analyzed by TLC.

extracts prepared from COS cells transfected with vector alone or with the full-length construction possessed low but detectable dsRNA-specific AdD enzyme activity (Fig. 8C). By contrast, extracts from COS cells transfected to express K88 dsRBP(296–1226) possessed significantly higher dsRNA-specific AdD enzyme activity than extracts from cells transfected with vector alone (Fig. 8C), suggesting that the K88-encoded protein indeed possessed intrinsic dsRNA-specific AdD activity.

Curiously, the activity of the dsRNA-specific AdD from cells in culture was previously reported to be only nuclear and unregulated by IFN (70). We therefore examined nuclear extracts prepared from both human U and SH-SY5Y cells for IFN-inducible dsRNA-specific AdD activity. Nuclear extracts prepared from human amnion U cells possessed significant dsRNA-specific AdD activity, and the activity was comparable in untreated and IFN-treated U cells (data not shown). By contrast, IFN-treated SH-SY5Y cells clearly showed elevated levels of dsRNA-specific AdD activity relative to untreated cells, as illustrated by the results shown in Fig. 9. However, quantitation revealed that the approximately 2-fold increase in dsRNA-specific AdD enzyme activity in IFN-treated SH-SY5Y cells was much less than the 10-fold or greater increase in p150 expression observed following IFN treatment (Fig. 3).

DISCUSSION

IFNs modulate numerous cellular functions, including the host response to viral infection (37, 50, 55). One approach to elucidating the physiological roles of IFN and the underlying mechanisms of action is to identify and characterize genes that encode IFN-regulated proteins. K88 is a cDNA clone that was isolated from a screening strategy designed to identify IFN-regulated human proteins. Several important and novel points

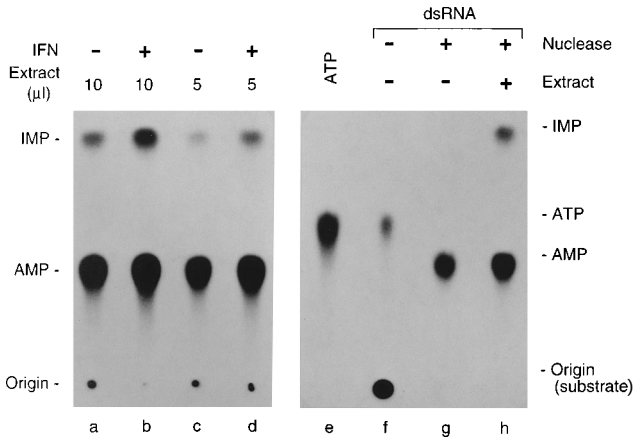

FIG. 9. IFN-inducible dsRNA-specific AdD enzyme activity. Nuclear extracts prepared from human neuroblastoma SH-SY5Y cells, either untreated (−) or IFN treated (+), were analyzed for AdD activity with ^{32}P-labeled dsRNA substrate. Different volumes (5 or 10 μl) of nuclear extract (1 μg of total protein per μl) were incubated with dsRNA substrate in the standard reaction mixture. Following digestion with P1 nuclease, the labeled nucleotides were analyzed by TLC. The positions to which the adenosine (AMP) and inosine (IMP) 5′-nucleotide monophosphates migrate, as well as the origin, are indicated. Controls for the analysis included ATP and the dsRNA substrate without incubation in the reaction mixture. Unlabeled nucleotides used as chromatography standards were visualized with 254-nm UV light.

emerge from the results reported herein on the cDNA structure, regulation, and expression of the K88 cDNA which encodes a double-stranded RNA-specific AdD.

K88-encoded protein is a novel IFN-regulated dsRNA-binding protein. The K88 cDNA hybridized to a single major RNA of 6.7 kb which was about fivefold more abundant in IFN-treated human cells than in untreated cells (Fig. 2). Immune sera generated against three different regions of the 1,226-aa ORF of the K88 cDNA expressed in *E. coli* recognized a protein of ~150 kDa apparent molecular mass (p150), which was increased about 5- to 10-fold by IFN treatment (Fig. 3 and 4). Polyclonal antibody prepared against the N-terminal region of the K88 cDNA (K88 Ab 3) was monospecific and recognized only the p150 protein by Western blotting. K88 Ab 3 recognized the p150 protein in IFN-treated human cell lines but not in mouse cells. Surprisingly, immune sera generated against either the central (K88 Ab 2) or the C-terminal (K88 Ab 1) region of the K88 ORF product recognized not only the IFN-inducible p150 protein but also a constitutively expressed protein of about 110 kDa (p110) in human cells. Although neither K88 Ab 3 nor 2 cross-reacted with p110- or p150-like mouse proteins, the K88 Ab 1 serum recognized an IFN-inducible mouse protein of ~150 kDa. The kinetics of induction of the 6.7-kb transcript and the p150 protein in human cells were comparable and similar to those observed for the IFN-inducible RNA-dependent protein kinase PKR in human cells (66). The IFN-inducible p150 protein likely corresponds to the primary translation product of the K88 cDNA.

Both the IFN-inducible p150 protein and the constitutively expressed p110 protein from human cells bound to double-stranded but not to single-stranded RNA-Sepharose affinity columns. The amino acid sequence deduced from the K88 cDNA includes three copies of the highly conserved R motif commonly found in dsRNA-binding proteins (41, 64). Recombinant GST-K88 fusion proteins from the central region of the cDNA ORF that included all three copies of the R motif possessed dsRNA-binding activity, whereas those that had only one copy bound dsRNA poorly, and dsRNA-binding activity was not detected in the absence of R motifs. These results are similar to those obtained for PKR, in which the R motif is repeated twice (22, 41, 64). In PKR, the two R copies differ quantitatively in their relative importance, both copies being required for maximal RNA-binding activity (23, 42).

Cell fractionation and indirect immunofluorescence microscopy studies revealed that the p150 protein was present in both the cytoplasm and the nucleus, whereas the p110 protein appeared primarily in the nucleus. The distribution of p150 thus, in some ways, parallels that of PKR. Although we showed herein that the IFN-inducible PKR kinase was found predominantly in the cytoplasm, as expected (56), when samples were analyzed in parallel for p150, p110, and PKR, some PKR was also detected in the nuclear fractions. Detection of a minor amount of the total PKR in nuclear fractions by Western blotting is in agreement with the results of immunofluorescence studies (29). These results suggest that the K88 cDNA-encoded product is a dsRNA-binding protein and furthermore that the IFN-inducible p150 protein and the constitutively expressed p110 protein are closely related dsRNA-binding proteins localized to different subcellular regions.

K88-encoded protein is an IFN-regulated dsRNA AdD. The 6,474-nt cDNA sequence that we had obtained for the K88 cDNA was very nearly identical to that recently reported for a dsRNA-specific AdD (33, 48). Expression of the K88 cDNA was IFN regulated (Fig. 2 to 4), and IFN treatment of human SH-SY5Y cells increased the level of dsRNA-specific AdD activity present in cell extracts (Fig. 9). Furthermore, both the full-length cDNA (33) and the cDNA deletion mutant lacking the N-terminal region but including aa 296 to 1226 (Fig. 8) produced dsRNA AdD activity when expressed in Sf9 cells and COS cells, respectively. By contrast, deletion of the C-terminal region from the cDNA abolished production of the enzymically active AdD in Sf9 cells (33). In addition, polyclonal antibody generated against the central region of the bovine deaminase cDNA clone blocked enzymic activity of purified bovine dsRNA-specific AdD (48).

These results suggest that the K88 cDNA-encoded protein is a dsRNA AdD and that this form of dsRNA AdD is IFN inducible. Furthermore, the results suggest that the catalytic domain of the dsRNA-specific AdD is localized to the C-terminal portion of the K88 protein. The C-terminal region of K88 displays striking sequence identity with the *C. elegans* T20H4.4 and *S. cerevisiae* HRA400 gene products and the mouse Tenr protein (Fig. 1B). The T20H4.4, HRA400, and Tenr proteins may well be deaminase homologs of the K88 dsRNA-specific AdD. If this notion is correct, then it seems likely that the *C. elegans* and *S. cerevisiae* counterparts, T20H4.4 and HRA400, may encode deaminases that are not selective for dsRNA, as the sequences lack the highly conserved dsRNA-binding R motif. By contrast, the Tenr protein includes a single copy of the R motif.

At least two forms of dsRNA AdD likely exist. Comparison of the biochemical properties and subcellular fractionation localizations of the K88 cDNA-encoded AdD proteins and dsRNA-specific AdD enzymatic activities from animal cells suggest that at least two forms of dsRNA-specific AdDs likely exist. The K88 cDNA clearly encodes an IFN-induced protein; probes from three different regions of the cDNA hybridized to a single major transcript that was IFN inducible (Fig. 2); and the K88-specific immune sera generated against three different regions of the 1,226-aa ORF recognized an IFN-inducible 150-kDa protein (p150) that was found in both the cytoplasm and the nucleus of human cells (Fig. 3 to 5). The cDNA also encoded a dsRNA-specific AdD enzymatic activity when expressed in monkey COS (Fig. 8) and insect Sf9 (33) cells.

Somewhat surprisingly, in the context of the IFN-inducible nature of the K88 6.7-kb mRNA and p150 protein, untreated human U and SY5Y cells displayed a high basal level of dsRNA-specific AdD enzyme activity. Although dsRNA-specific AdD activity was enhanced by IFN treatment in the case of human SH-SY5Y cells, human U cells did not show an IFN-mediated increase in AdD activity even though p150 induction was readily observed in U cells. These findings are in agreement with an earlier report that the dsRNA-specific deaminase was not induced by IFN (70). Curiously, the dsRNA-specific deaminase was previously reported to be a nuclear enzyme (32, 70). Relevant to this earlier observation is our finding that, in addition to p150, K88-specific sera raised against the C-terminal region of the cDNA ORF predicted to represent the catalytic domain (K88 Ab 1) or the central region (K88 Ab 2) also recognized an abundant and constitutively expressed 110-kDa protein that localized exclusively to the nucleus.

Our localization by immunofluorescence microscopy of the p110 protein to the nucleus of untreated human U and SH-SY5Y cells is similar to a recent report for untreated HeLa cells (48). Antibody prepared against the region of the bovine cDNA clone product corresponding approximately to the same region that we used to generate Ab 2 against the human cDNA product recognized a protein of about 116 kDa in extracts prepared from HeLa cells that had not been treated with IFN (48). The lack of cytoplasmic staining with untreated HeLa cells and the lack of detection of the p150 protein in cell extracts prepared from untreated HeLa cells (48) are consistent with our finding that the p150 cytoplasmic protein is induced by IFN. p150 was not readily detectable in either U or SY5Y cells in the absence of IFN treatment.

The size of the protein predicted from the ORF of the K88 dsRNA-specific AdD cDNA is 136 kDa (Fig. 1). The IFN-inducible p150 protein detected in human cells comigrated on SDS-PAGE with the product (136 kDa) of the K88 cDNA expressed in transfected COS cells (Fig. 8). By contrast, the dsRNA-specific AdD enzymatic activity purified from cells is uniformly described as a much smaller protein, one which is more comparable in size to the constitutively expressed p110 nuclear protein detected with K88 Ab 1 and 2. dsRNA-specific AdD sizes are 83 to 93 kDa for the bovine liver enzyme (32), 116 kDa for the calf thymus enzyme (47), and 120 kDa for the *Xenopus* enzyme (27). Biochemical and physiochemical analyses indicate that the various forms of dsRNA AdD protein all possess the same enzymatic activity. It is conceivable that the dsRNA-specific enzyme preparations that have so far been purified (27, 32, 47) correspond to the more abundant and constitutively expressed form of dsRNA-specific AdD, perhaps the p110 protein. Our results imply that the IFN-induced p150 protein, which appears to correspond to the full-length product of the K88 dsRNA-specific AdD cDNA, represents a novel dsRNA-specific AdD, one that is IFN regulated and much less abundant that the constitutive enzyme. Indeed, RNA competition and column fractionation studies indicated that the GluR-B pre-mRNA and the dsRNA-specific AdD activities are distinct (73).

Functional significance of an IFN-induced dsRNA AdD. dsRNA-specific AdD, as an RNA editing enzyme, catalyzes the C-6 deamination of adenosine to yield inosine (9, 69). Such a covalent modification of viral dsRNA molecules could greatly impact the efficiency of the viral multiplication process, for example, by altering either the sequence or stability of duplex RNA replicative structures or by changing mRNA coding properties. Because the previously characterized dsRNA-specific AdD is a nuclear enzyme, it is conceivable that the IFN-induced K88-encoded cytoplasmic deaminase is the form that plays a central role in the host response to viral infection, especially for viruses that replicate in the cytoplasm. Thus, an IFN-induced dsRNA-specific AdD could contribute to determining the outcome of a viral infection, that is, whether the virus is cleared or a persistent infection is established.

Among the enzymatic activities that are implicated in the antiviral actions of IFN, two of the best characterized are dsRNA-binding proteins: PKR, the kinase which inhibits translation by phosphorylation of protein synthesis initiation factor eIF-2α (62), and the 2′,5′-oligoadenylate synthetase, whose oligonucleotide product activates an RNase which degrades mRNA as well as rRNA (62). The PKR kinase and the 2′,5′-oligoadenylate synthetase-nuclease both cause a reduction in viral protein production, albeit by quite different biochemical mechanisms (50, 55, 62). Possibly the dsRNA-specific AdD likewise mediates a reduction in the production of viral proteins by virtue of the A-to-G (U-to-C) RNA editing that can introduce premature translation termination codons in viral transcripts (6, 16, 17). If indeed the dsRNA-specific AdD is proven to be responsible for the production of mutated viral mRNAs that cannot be translated to yield functional protein (10, 15), this effect of IFN-enhanced RNA editing would then establish a third mechanism by which IFN directly affects the synthesis of viral proteins.

There has been considerable interest in the potential therapeutic efficacy of the IFNs in the treatment of certain central nervous system diseases, for example, multiple sclerosis and SSPE. Clinical trials with IFN-β in the treatment of multiple sclerosis patients have met with considerable success (4). By contrast, the potential efficacy of IFNs or their inducers for SSPE, a fatal disease caused by infection with measles virus in the brain (13), gave mixed results (28). Because a dsRNA-specific AdD is implicated as the cause of the biased hypermutations observed in measles virus transcripts in SSPE patients (13, 16), enhanced levels of cytoplasmic dsRNA-specific AdD might therefore facilitate SSPE as measles virus replicates in the cytoplasm (20). Although IFNs have potent antiviral activities both in cell culture and in animals (50, 55), they paradoxically have been implicated in helping to establish persistent viral infections (61). Here again, the molecular basis of an IFN-facilitated development of a persistent infection may reside in the IFN-induced cytoplasmic dsRNA-specific AdD. Indeed, dsRNA-specific AdD is presumed to be the cause of the hypermutations observed during persistent infections by various RNA viruses with cytoplasmic multiplication cycles, including vesicular stomatitis virus, parainfluenza virus, and measles virus (10, 46, 49).

Recent studies have shown that functional expression of the family of glutamate receptor subunits in the brain is controlled in part by RNA editing (26, 38). Editing occurs in >99% of the Q/R sites in murine brain and neuronal cell lines, including SH-SY5Y cells (26), and has been suggested to result from a dsRNA AdD activity in the nucleus. The cell can control this in a site-specific manner because intron sequences are required to form a corresponding dsRNA structure that only occurs in the nucleus prior to splicing. Because of the high level of basal deaminase activity, it seems unlikely that the relatively low level of expression of K88 dsRNA AdD p150 protein would be responsible for the GluR pre-mRNA deamination. Perhaps the abundant p110 nuclear protein is a more likely candidate for a housekeeping type of dsRNA-specific AdD.

Many viruses have devised strategies to antagonize the antiviral action of IFN, often by impairing the function of the RNA-dependent protein kinase PKR (40, 56). Vaccinia virus, for example, encodes two proteins that impair PKR function:

K3L, which is an eIF-2α substrate analog, and E3L, which is an RNA-binding protein that is believed to function by competing with PKR for the binding of activator RNAs. Curiously, we identified two separate regions in the K88-encoded dsRNA AdD that displayed sequence similarity to the vaccinia virus E3L protein. One region was the dsRNA-binding R motif, but the other region was the N-terminal region of E3L, which is not necessary for dsRNA-binding activity (18). The functional significance of this homology remains to be established. Although E3L was found associated with viral factories in the cytoplasm, the protein was primarily found in the nucleus of transfected and infected cells (74). E3L may modulate the activity of the basal nuclear dsRNA AdD enzyme, and perhaps also cytoplasmic AdD, in a manner conceptually similar to that envisioned for antagonism of the cytoplasmic PKR.

It is now of utmost importance to characterize in biochemical terms the dsRNA-specific cytoplasmic p150 AdD from IFN-treated cells and the nuclear p110 enzyme from untreated cells and to attempt to identify the viral and cellular RNAs that are capable of serving as substrates for dsRNA-specific AdD enzymes in the hope of gaining further insights about the biological processes modulated by them.

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