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Molecular Cloning of Two New Interferon-induced, Highly Related Nuclear Phosphoproteins*

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During the molecular cloning of the human dsRNA activated-p68 kinase (PKR), polyclonal antibodies against PKR selected, in addition to cDNAs corresponding to PKR, another cDNA presenting only slight homology with PKR cDNA. This cDNA recognized an mRNA species of 2 kilobases induced by both α- and γ-interferons. Its transcription did not require protein synthesis. On further library screening, it selected two highly related cDNAs, referred to as 75 and 41, displaying perfect homology over 612 base pairs and divergent at both ends. In addition, cDNA 75 presents an insertion of 150 base pairs highly homologous to a region common to both sequences. The 75 and 41 peptidic sequences are very hydrophilic, rich in basic amino acid residues, and contain several potential phosphorylation sites for different serine/threonine kinases. Furthermore, they present two protamine- and histone-like nuclear targeting sequences as well as some homology with helix-loop-helix motifs of some DNA-binding proteins. The 75-encoded product, which resolved as a 52-kDa protein after *in vitro* expression in rabbit reticulocyte lysates, was found to migrate as a 65–67-kDa protein after *in vivo* expression in insect cells. In accord with sequence data, this 65–67-kDa protein was found to be phosphorylated *in vivo* in the insect cells and was recovered from the membrane/nuclear pellet. In contrast, the 41-encoded product (30-kDa protein in reticulocyte lysates) could not be expressed *in vivo*, as it provoked a rapid and severe shut-off of protein synthesis in insect cells. The function of the 75 and 41 proteins and their relation to PKR remains to be determined. However, the presence of nuclear targeting sequences, phosphorylation sites, and helix-loop-helix motif is consistent with a role of these proteins in the mechanism of transduction of the interferon action.

Interferons (IFNs)[1] are a family of proteins involved in a number of regulatory functions such as control of virus growth, control of cell proliferation, and regulation of immune functions and differentiation. This family can be subdivided into three classes of molecules, IFN-α, IFN-β, and IFN-γ, which exert their pleiotropic actions after binding to closely related receptors for IFNs -α/-β (type I receptors) (Merlin *et al.*, 1985; Uze *et al.*, 1990) and to an unique receptor for IFN-γ (type II receptor) (Aguet *et al.*, 1988). In response to interferon action, a large number of cellular genes can be induced, depending on the interferon species and the cell type, a process that can be attributed both to specific responsive elements in the promoter regions of interferon-responsive genes and to the presence and activation of specific transactivation factors (Friedman and Stark, 1985; Levy *et al.*, 1990; Fu *et al.*, 1992). Among the different interferon-induced proteins, some have been clearly related to the antiviral action of interferon (such as the Mx proteins (Staeheli *et al.*, 1986) and the two dsRNA-activated enzymes), the 2–5A synthetases, and the p68/p65 kinase (Chebath *et al.*, 1987; Coccia *et al.*, 1990; Meurs *et al.*, 1992). The human dsRNA-activated p68 kinase (now simply referred to as PKR for protein kinase dsRNA-dependent) proved, in addition to its antiviral function, to manifest an antiproliferative and a potential tumor suppressor function (Chong *et al.*, 1992; Koromilas *et al.*, 1992; Meurs *et al.*, 1993). In relation to the IFN immune functions, both α-/β- and γ-IFNs enhance class I histocompatibility antigens. In addition, γ-IFN increases class II histocompatibility antigens, thus improving the antiviral cellular immune process by favoring effector-immune T cell recognition of viral cell surface antigens (Amaldi *et al.*, 1989; Fellous *et al.*, 1982; Wallach *et al.*, 1982).

The unraveling of the complex interferon network requires the cloning of already characterized IFN-induced proteins as well as systematic search for new IFN-induced genes. Several IFN-induced genes have been isolated over the past 10 years, using different techniques. For instance, differential screening of cDNA libraries from IFN-treated or untreated cells yielded the 40–46 kDa 2–5A synthetase (Merlin *et al.*, 1983), an abundant p56 protein of still unknown function (Chebath *et al.*, 1983; Larner *et al.*, 1984), and several proteins induced by γ-IFN in macrophages: IP10 (homologous to platelet factor 4 and β-thromboglobulin) (Luster *et al.*, 1985); IP30 (Luster *et al.*, 1988), and 4-kDa γ-1 (Fan *et al.*, 1989). Use of oligonucleotides derived from purified peptides yielded the IFN-β-induced ubiquitine homolog 15-kDa protein (ISG15) (Korant *et al.*, 1984; Blomstrom *et al.*, 1986; Haas *et al.*, 1987; Loeb and Haas, 1992) and an IFN-γ-induced 55-kDa protein homologous to rabbit peptide chain release factor and to bovine tryptophanyl tRNA synthetase (Fleckner *et al.*, 1991). Detailed analysis of the mechanism of IFN and IFN-induced gene induction established that IFN-β and IFN-γ can, respectively, induce the synthesis of the transcription factors

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L22342 (41, 1045 bp) and L22343 (75, 1542 bp).

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[1] The abbreviations used are: IFN, interferon; PKR, human dsRNA-activated p68 kinase; kb, kilobase(s); bp, base pair(s); HLH, helix-loop-helix; PAGE, polyacrylamide gel electrophoresis.

IRF1 (44 kDa; involved in IFN-β transcription) (Harada et al., 1989) and ISGF3-γ (48 kDa; involved in the transcription of most of the IFN-induced genes) (Levy et al., 1990; Veals et al., 1992). Screening of expression libraries with antibodies specific for purified IFN-induced proteins allowed the cloning of the human p68 PKR (Meurs et al., 1990) and of the 69-kDa 2-5A synthetase (Marie and Hovanessian, 1992). It also yielded again the tryptophanyl tRNA synthetase (Rubin et al., 1988; Rubin et al., 1991).

Interestingly, during the screening of the cDNA for the p68 PKR with the use of specific antibodies, we isolated another cDNA corresponding to an interferon-induced mRNA but completely unrelated to the p68 PKR cDNA as shown by its nucleotide sequence analysis. Here, we report its cloning and sequence, which leads to the characterization of two new interferon-induced proteins with nuclear targeting domains and phosphorylation sites, which may be substrates for serine/threonine kinases.

EXPERIMENTAL PROCEDURES

Cells—Daudi cells were grown in suspension in RPMI 1640 medium containing 10^{-5} M 2-mercaptoethanol and 10% fetal calf serum. HeLa cells were grown in monolayer cultures in Dulbecco's medium containing 10% fetal calf serum. Insect *Spodoptera frugiperda* (SF9) cells were grown in TC100 medium containing 10% fetal calf serum.

Isolation of cDNA Clones—Oligo(dT) and random-primed cDNA λgt11 and λgt10 libraries were constructed from human α-IFN-treated Daudi cells as described (Meurs et al., 1990). The oligo(dT)-primed cDNA λgt11 Daudi cell library was immunoscreened using human PKR-specific polyclonal antibodies. After four rounds of immunoscreening, antibody-positive clonal plaques yielded a cDNA of 830 bp, referred to as K1, hybridizing to an IFN-induced 2.5-kb RNA, which ultimately allowed the molecular cloning of PKR (Meurs et al., 1990). When cDNA K1 was used as a probe on different anti-PKR-positive plaques obtained from the third round of immunoscreening, it yielded (in addition to itself as expected) a different cDNA of 620 bp, referred to as 1.2, which hybridized to an IFN-induced 2-kb RNA species. This 1.2 fragment was then used as a probe to screen the random-primed cDNA λgt10 Daudi library. One clone was selected, which yielded an insert of 1044 bp (insert 41) corresponding to the 1.2 sequence but extended at its 5′ end. By Northern blot analysis, this 41 cDNA hybridized to the IFN-induced 2-kb RNA species but, in addition, hybridized to an abundant constitutive 0.5-kb RNA species. The cDNA 41 was subcloned into the *Eco*RI site of Bluescript KSM13$^+$, and different fragments were generated by restriction enzyme digestion. Further Northern blot analysis with these different fragments showed that 0.5-kb RNA hybridized only to the 5′ end of cDNA 41 (*Pst*I-*Ppu*MI fragment of 393 bp). In order to avoid the selection of cDNAs partially homologous to the 5′ end of 41 cDNA and to be able to extend the 41 cDNA at its 3′ end, a differential screening of the initial cDNA λgt11 Daudi library was then designed, using the *Pst*I-*Ppu*MI fragment, the 41 cDNA, and the 1.2 cDNA. By systematically discarding plaques hybridizing to the *Pst*I-*Ppu*MI probe and amplifying those hybridizing only to both 1.2 and 41 probes, we finally obtained one clone containing an insert of 1540 bp (referred to as 75), which hybridized only to the interferon-induced 2-kb mRNA.

DNA sequences were determined by the Sanger dideoxy sequencing method (Sequenase, U. S. Biochemical Corp.). Sequence analysis was performed with the Genejockey program (MacIntosh). Search for sequence homologies was performed using the Genbank database.

RNA Analysis—For separation of cytoplasmic and nuclear RNAs, cells were pelleted, washed with phosphate-buffered saline, resuspended in 1 ml of lysis buffer (0.14 M NaCl; 1.5 mM $MgCl_2$; 0.05% Nonidet P-40; 1000 units/ml RNasin; 10 mM Tris, pH 8.6), layered over 1 ml of lysis buffer containing 24% sucrose and 1% Nonidet P-40, and centrifuged at 8000 rpm for 30 min at 4 °C. The cytoplasmic RNAs (upper phase) and the nuclear RNAs (pellet) were then extracted as follows: 5 ml of buffer I (6 M guanidium thiocyanate (Fluka), 5 mM EDTA, 0.1 M sodium acetate, pH 5.2) was added, and the suspension was layered over 5 ml of solution II (5 M cesium chloride; 5 mM EDTA, 0.1 M sodium acetate, pH 5.2) and centrifuged at 31,000 rpm for 15 h in an RPS 40T-789 rotor of an LKB ultracentrifuge. The RNA pellet was resuspended in water and precipitated twice with ethanol at −20 °C before gel analysis. Extraction of total cellular RNA was performed by directly resuspending cell pellets in 6 ml of solution I before centrifugation over 5 ml of Solution II. Electrophoresis, transfer, probe preparation, and hybridization were performed by standard techniques (Sambrook et al., 1989). For run-on analysis, preparation of nuclei from Daudi cells, transcription in isolated nuclei, and hybridization of ^{32}P-labeled RNAs were carried out as described (Meurs and Hovanessian, 1988).

In Vitro Transcription—The 41 and 75 cDNAs were excised by *Eco*RI digestion from Bluescript KSM13$^+$, blunt-ended, and subcloned in plasmid pcDNAI/NEO (Invitrogen) at the *Eco*RV site of the polylinker region. For transcription of full-length mRNAs, the 75 pcDNA1/NEO and the 41 pcDNA1/NEO were linearized with *Xho*I. The recombinant p68-pcDNA1/NEO, encoding the human PKR, was linearized with *Bam*HI. Transcription from the T7 promoter in the presence of the cap analog 7-mGpppG was carried out using the mRNA capping kit from Stratagene.

In Vitro Translation—The *in vitro* transcribed mRNAs were translated in rabbit reticulocyte lysate (Amersham Corp.) according to the manufacturer's instructions using 30 μg/ml template RNA in presence of 2O μCi of [^{35}S]methionine in a 50-μl reaction volume. An equal volume of 2 × protein electrophoresis buffer was added and the products analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

Expression in the Baculovirus System—The 41 and 75 cDNAs were cloned into the *Eco*RI site of the transfer plasmid pVL1393 (Invitrogen), and 0.5 μg of each cotransfected into SF9 cells with 0.1 μg of "Baculogold" virus DNA (AMS Biotechnology, United Kingdom) using the "Transfectam" reagent (Promega). Recombinant progeny virus was plaque-purified twice on monolayers of SF9 cells (Summers and Smith, 1987) in the presence of X-Gal (10 μg/ml, Life Technologies, Inc.), non-recombinant background virus being identified by a blue plaque morphology. The identity of purified virus isolates was verified by polymerase chain amplification of total DNA from infected cells (Malitschek and Schartl, 1991) and by analysis of expressed proteins (see below).

To analyze expression of recombinant proteins, monolayers of SF9 cells were infected at an multiplicity of infection of 10 plaque-forming units/cell. Virus was allowed to adhere for 1 h at 28 °C before cells were overlayed with fresh culture medium. Plates were incubated for up to 3 days before harvesting. For Coomassie Blue staining of proteins, monolayers were rinsed twice with phosphate-buffered saline, scraped off, and collected by centrifugation. For isotopic labeling, 3-day infected cells were overlayed with culture medium either lacking both methionine and cysteine and containing both amino acids labeled with Tran^{35}S-label (ICN, 100 μCi/ml final) or with medium lacking phosphate and containing 50 μCi/ml [^{32}P]orthophosphate (carrier-free, Amersham). After a 3–4-h labeling period at 28 °C, monolayers were rinsed and harvested as above. All cell pellets were lysed in lysis buffer (150 mM NaCl, 1.5 mM $MgCl_2$, 0.65% Nonidet P-40, 10 mM Tris-HCl, pH 7.9) and either mixed with an equal volume of SDS-electrophoresis buffer or fractionated into a nuclear/particulate fraction by centrifugation at 4000 rpm before addition of SDS buffer. Proteins were analyzed by electrophoresis by SDS-PAGE on 1O% polyacrylamide gels.

RESULTS

Isolation of a New Interferon-induced cDNA with Antibodies Specific for the p68-PKR—The cloning of the cDNA encoding the p68 PKR was originally started by screening a λgt11 library expressing IFN-induced Daudi oligo(dT)-primed cDNAs with p68-PKR specific polyclonal antibodies. This yielded an 831-bp cDNA (K1) insert hybridizing to an IFN-induced 2.5-kb mRNA, which was further identified as the p68-PKR cDNA (Meurs et al., 1990). In addition, this immunoscreening yielded another insert cDNA of 620 bp, referred to as 1.2, which hybridized to another interferon-induced mRNA of 2 kb. Isolation of such an interferon-induced cDNA using a specific anti-PKR antibody was interesting enough to determine its relation to p68-PKR. On first analysis, however, no striking homology was found between the 1.2 and the PKR sequences, and a computer search for homology in protein sequence data did not reveal strict identity between 1.2 and already described proteins. Further char-

acterization of this new IFN-induced cDNA was therefore investigated.

The kinetics of expression of the RNAs hybridizing to this insert 1.2 following interferon treatment was analyzed both at the transcriptional and at the RNA level, in parallel with those of the PKR mRNA (Fig. 1, *A* and *B*). The kinetics of induction of both genes were found to be similar. Like the transcription of the PKR (K1 probe), the transcription of the 2-kb RNA (1.2 probe) was rapidly induced by interferon at the transcriptional level and was not dependent on continuous protein synthesis, since it was unaffected by cycloheximide treatment of cells (Fig. 1*A*). On Northern blots, the 1.2 probe hybridized strongly to the 2-kb species (Fig. 1*B*). In addition, it hybridized also to 6, 2.5, and 1.8-kb RNA species. The 6-kb RNA may represent a nuclear precursor form, whereas the 2.5-kb RNA most probably represents cross-hybridization with the PKR mRNA, since, reciprocally, the PKR K1 probe could hybridize with the 2-kb species. This latter is in contradiction with the absence of apparent homology between the 1.2 and the PKR sequences (see "Discussion"). The 1.8-kb RNA species did not represent nonspecific hybridization to the 18 S ribosomal species, as it was clearly induced by interferon. The nature of this 1.8-kb mRNA is discussed below.

We next analyzed the specificity of induction of the 2-kb mRNA in response to IFN-α or IFN-γ. Since Daudi cells do not respond to IFN-γ treatment, HeLa cells were treated with IFN-α or with IFN-γ, and total RNA was extracted and analyzed in Northern blots (Fig. 1*C*). Hybridization to an actin probe, used as control, showed that the actin levels were not affected by interferon treatment. Hybridization to the 1.2 probe showed that both types of interferon can induce the 2-kb mRNA levels in HeLa cells and that IFN-γ was a better inducer than IFN-α.

Cloning of Two cDNAs Hybridizing to the IFN-induced 2-kb mRNA—The fragment 1.2 was used as a probe to isolate additional clones from Daudi and HeLa cDNA libraries. First, a cDNA of 1044 bp, referred to as 41, was isolated from the initial Daudi library. In Northern blot, this cDNA hybridized to the IFN-induced 2-kb mRNA but hybridized in addition to an abundant 0.5-kb mRNA species present at a constitutive level in both control and interferon-treated cells (Fig. 2*A*). The nucleotide sequence of 41 was found to be identical to that of 1.2 but extended at its 5′ end by 415 nucleotides. Recognition of the 0.5-kb RNA was attributed to hybridization with part of this region (see "Experimental Procedures"). In order to extend the 41 sequence in its 3′ end and to avoid further selection of uninduced cross-reacting cDNAs, a screening was devised to select plaques hybridizing only to 1.2 and 41 probes (hybridizing to 2-kb mRNA) but not to the 5′ end of cDNA 41 (hybridizing to 0.5-kb mRNA) (see Fig. 2*B*). This screening yielded a cDNA of 1540 bp, which, in Northern blot, hybridized only to the IFN-induced 2-kb mRNA species and not to the 0.5-kb RNA (Fig. 2*A*). This cDNA was referred to as cDNA 75. Sequence analysis confirmed the Northern data but showed that 41 and 75 cDNAs were different, though highly related (see below). Thus, we have isolated not one but two different cDNAs corresponding to the interferon-induced 2-kb mRNA species. In that respect, it is possible that the 1.8-kb species, which can be detected in some gels, represent one sequence and the 2-kb species the other one.

Sequence Analysis of 75 and 41 cDNAs—The complete nucleotide and amino acid sequences of the 75 and 41 cDNAs are presented in Fig. 3*A*. The sequence of 75 cDNA predicts an open reading frame of 1113 bp corresponding to a polypeptide of 371 amino acids, while the sequence of 41 cDNA predicts an open reading frame of 744 bp corresponding to 248 amino acids. Predicted molecular weights are 42,221 and 28,591 daltons for polypeptides encoded by 75 and 41 cDNAs, respectively. Two important characteristics of these cDNAs can immediately be drawn from their comparison; first, they present a region of strict homology over 612 nucleotides (204 amino acids residues) (Fig. 3*A*, *dashed line*) and second, this region of homology is broken in the 75 sequence by a 150-bp insert. The 75 and 41 cDNA sequences differ at both ends (385 bp specific for 75 and 282 bp specific for 41 at their 5′ end; 390 bp specific for 75 and 146 bp specific for 41 at their 3′ end). Their homology starts at nucleotide 386 for 75 cDNA and nucleotide 283 for 41 cDNA. The 150-bp insert is located after nucleotide 456 on the 75 cDNA sequence (corresponding to nucleotide 354 on the 41 sequence). At nucleotide 609 (75) and 355 (41), homology resumes until nucleotide 1150 (75) and 898 (41). A dot matrix graphic plot drawn from the protein sequence clearly visualizes the region of homology as well as the brake point where the insertion occurs (Fig. 3*B*). Moreover, this plot shows that the insert found in 75 cDNA sequence presents a strong homology with a region downstream of the brake point and therefore common to both 41 and 75 cDNAs. Further comparison of the insertion (residues 60–109 of 75 sequence) with the brake point downstream region (residue 76–126 of 41 sequence) shows 66% homology between the two sequences at the amino acid level, which increases to 88% homology when conservative substitutions are considered (Fig. 3*A*, identical amino acid is *underlined*).

Presence of Several Putative Phosphorylation Sites on 41 and 75 Sequences—A hydropathy plot drawn from the sequences showed that they present highly hydrophilic structures with only one major hydrophobic region at their carboxyl-terminal region and that they are rich in basic amino acids (not shown). Closer analysis on their amino acid sequence reveals that 41 and 75 present several serine and threonine residues, often located near basic residues, which suggests that they could represent potential phosphorylation sites for different types of serine/threonine kinases, such as cGMP kinases (RCIWS, KKKS, RKS, TQRKDDST), cAMP kinases (RKS), protein kinase C (SSK, SVK), and cdc2 kinase (STPKRR). The position of these sites on the two sequences is *boxed*, except for the cdc2 kinase, which is *underlined*, in Fig. 3*A*. Particularly interesting is the sequence TQRKDDST, as it can be used according to four different combinations (RXXXS; KXXS; TXR; KXXXT), strongly suggesting potential cGMP kinase phosphorylation sites (Kemp and Pearson, 1990). The 41 and 75 sequences present eight potential phosphorylation sites in common, while the 75 sequence presents an additional three sites due to its 50-amino acid insertion. One of these three sites, a potential site for caseine kinase II (STVE), is unique to the 75 sequence.

The 41 and 75 Peptidic Sequences Present Properties of Nuclear Basic Proteins—A search for amino acid sequence homology in the Genbank database revealed no strict identity of the 75 and 41 sequences with already described proteins. However, in accord with their high percentage of basic residues, 75 and 41 predicted protein sequences presented two regions of homology, one (in the middle of their sequences) with some sheep and bovine histone proteins (32% homology) and the other (at the amino terminus) with some protamines (rainbow trout and chum salmon, 25% homology). Indeed, subsequent detailed analysis showed that the 75 and 41 proteins present several of the histone properties such as proline, serine, and basic residue-rich regions (for review, see Churchill and Travers (1991)). Some homology (15%) was also found

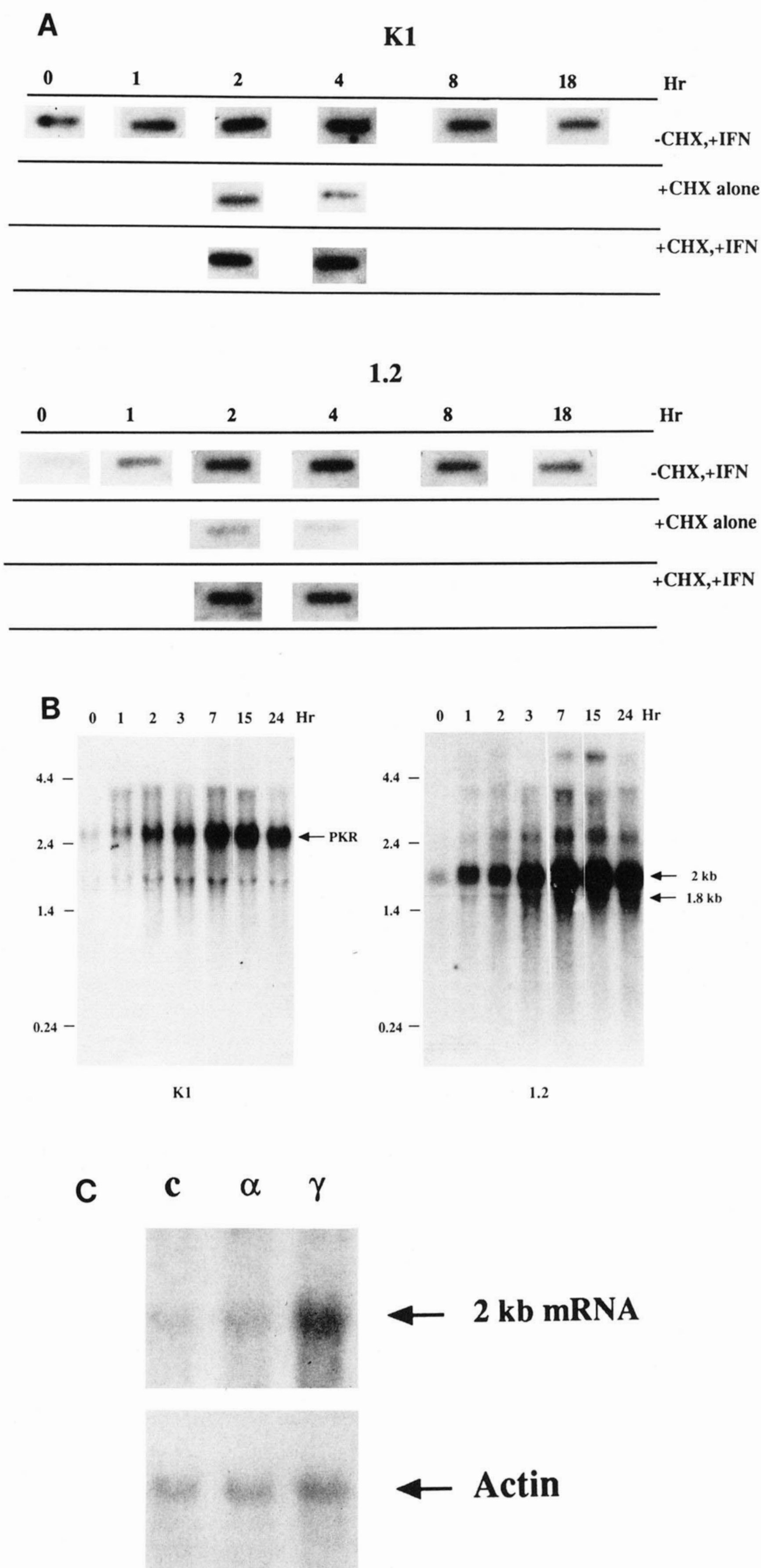


FIG. 1. *A*, transcription of the 2-kb RNA in Daudi cells. Blot assay of nuclear transcripts after treatment with interferon is shown. Daudi cells were incubated alone (*0*) or treated with 500 units/ml of IFN-α for 1, 2, 4, 8, and 18 h (*−CHX,+IFN*). For transcription assay during protein synthesis inhibition, Daudi cells were incubated with cycloheximide (50 μg/ml) for 20 min before the addition of 500 units/ml IFN-α (*+CHX,+IFN*) and further incubated for 2 and 4 h. As a control, cells were incubated in the presence of cycloheximide only (*+CHX alone*). RNAs from isolated nuclei labeled *in vitro*, with [γ-^{32}P]UTP were analyzed by hybridization with a linearized 1.2 cDNA-containing Bluescript plasmid, which was bound on Hybond-N (Amersham) membrane filters (see "Experimental Procedures"). K1 corresponds to a linearized Bluescript plasmid containing an 831-bp fragment from the PKR probe. *B*, kinetics of induction of the new 2-kb mRNA and the PKR 2.5-kb mRNA by IFN-α. Cytoplasmic RNAs was extracted from Daudi cells after 0, 1, 2, 3, 7, 15, and 24 h of treatment with 500 units/ml of IFN-α, separated by electrophoresis on 1% agarose gels (10 μg/lane), and analyzed by Northern blot hybridization successively with the 1.2 probe (620 bp) and the K1 PKR probe (831 bp). RNA species of 1.8, 2, and 2.5 kb (PKR) are indicated by *arrows*. The size (in kb) of RNA markers (Life Technologies, Inc.) are shown on the *left*. *C*, induction of the 2-kb RNA by IFN-α and IFN-γ. HeLa cells were treated with 500 units/ml of IFN-α or IFN-γ. Cytoplasmic RNAs (20 μg) were separated on 1% agarose gels and hybridized in Northern blots to the ^{32}P-labeled 1.2 cDNA. To control the specificity of the induction, the blot was dehybridized and rehybridized with a probe for actin, the levels of which are not affected significantly by interferon treatment.

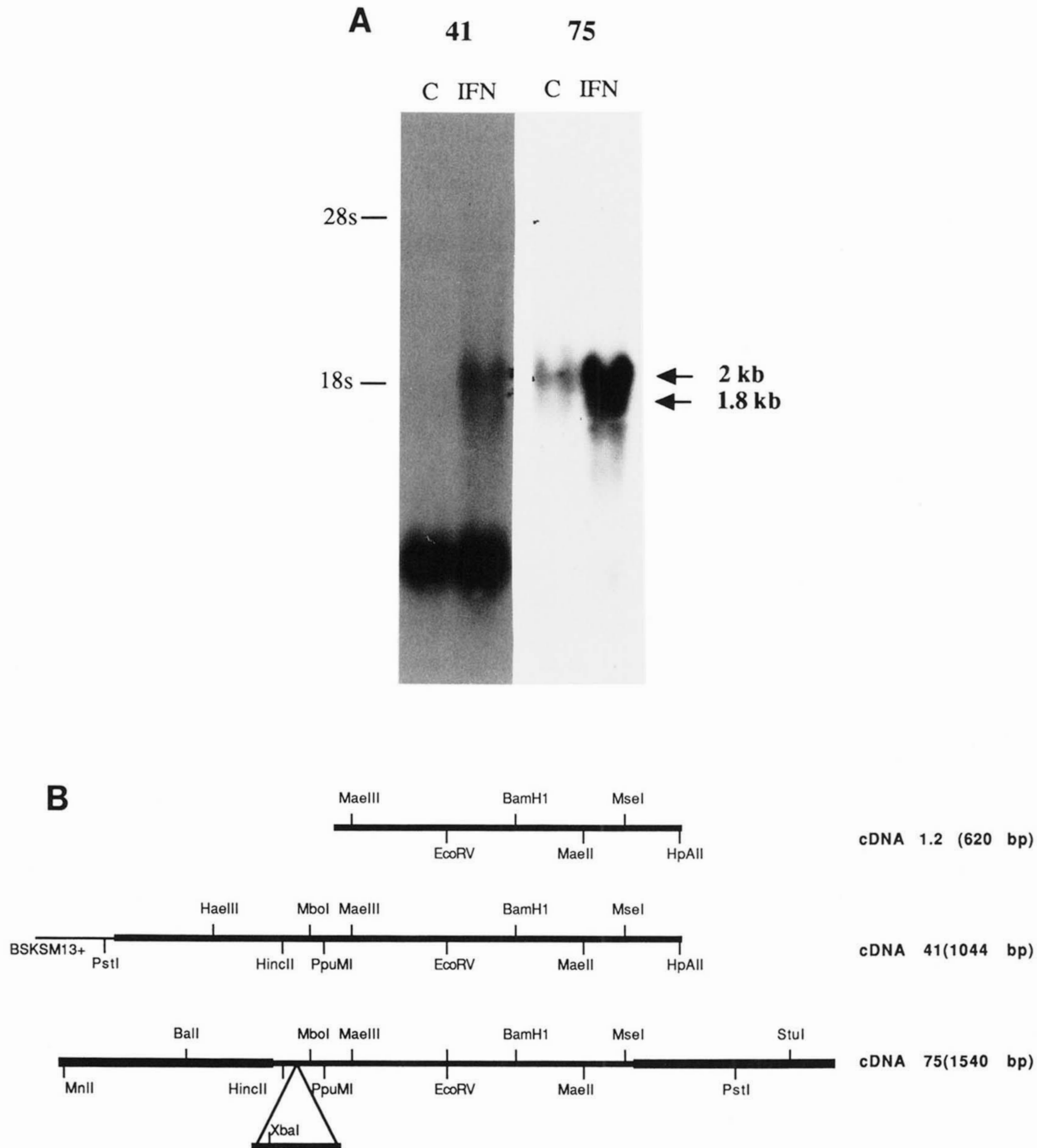


FIG. 2. *A*, selection of the cDNAs specific for the interferon-induced 2-kb RNA. Different Northern blots of cytoplasmic RNAs from Daudi cells, either untreated (*C*) or treated with 500 units/ml of IFN-α for 18 h (IFN), were hybridized to different cDNAs isolated from cDNA libraries as described under "Experimental Procedures." The interferon-induced RNAs species are shown by *arrows*. The position of the 28 and 18 S ribosomal RNA is indicated on the *left*. *B*, restriction endonuclease maps of the 1.2, 41, and 75 cDNAs. The cDNA 1.2 was initially isolated from an IFN-treated Daudi λgt11 expression library with anti-PKR antibodies. The cDNA 41 was then isolated from an IFN-treated Daudi λgt10 library using cDNA 1.2 as a probe. Fragment *Pst*I-*Ppu*MI was prepared after subcloning cDNA 41 in the Bluescript KS M13$^+$, vector using the *Pst*I site of the polylinker region of the plasmid and the internal unique *Ppu*MI site of cDNA 41. The *Pst*I-*Ppu*MI fragment was used as a probe in conjunction with the 1.2 and the 41 probes to differentially screen the IFN-treated Daudi λgt11 library. This yielded cDNA 75. The three cDNAs were aligned in this scheme to highlight their homologies (same restriction map) and their differences, represented by the *thicker black regions* and by the insert in the cDNA 75 sequence.

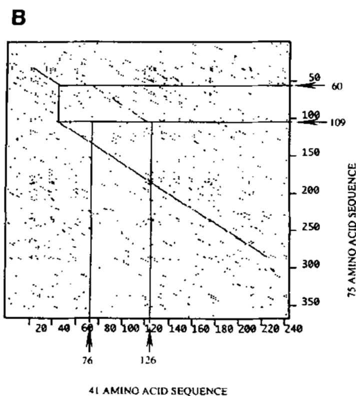
with a recently described arginine-rich nuclear protein of 54 kDa, which has been proposed to be involved in the pre-mRNA splicing pathway (Chaudhary *et al.*, 1991). The very rich basic residue content of 75 and 41 protein sequences and their homology with nuclear proteins prompted us to search for nuclear targeting motifs. Nuclear targeting sequences were described to consist of two discrete clusters of basic amino acids separated by any ten amino acids (Dingwall and Laskey, 1991). Two such motifs were found in the sequence common to both 41 and 75 (**KR**CIWSTPKRRH**KK** and **KK**KEK-DICSSSK**RR**FQK) (see Fig. 3*A*, *boldface type underlined*). Their presence suggest nuclear localization of the 75 and 41 encoded proteins, since the accumulation of the bipartite motifs favor the initial rate and final level of nuclear accumulation (Dingwall and Laskey, 1991). Interestingly, all the phosphorylation sites found in the 75 and 41 amino acid sequences are mainly located within or between the two putative nuclear targeting motifs, thus raising the possibility that their phosphorylation may serve to enhance the nuclear uptake of the 75 and 41 encoded proteins, as reported for oncogene products and transcription factors (Robbins *et al.*, 1991).

In Vitro Transcription/Translation of cDNAs 41 and 75—The 41 and 75 cDNAs were subcloned in pcDNA1/NEO vectors, linearized, and transcribed from their T7 promoter (see "Experimental Procedures"). The RNAs were then translated using the rabbit reticulocyte translation system. Transcription and translation of PKR (from pcDNA1/NEO-p68)

FIG. 3. *A*, nucleotide sequence and deduced amino acid sequence of 75 and 41. The nucleotide and amino acid sequences of 75 (1540 bp, 371 amino acids) and 41 (1044 bp, 248 amino acids) have been aligned on the same figure to highlight their homologies. Nucleotides are *numbered* as shown at the immediate *left* of the sequences. Amino acids are *numbered* above (41) and under (75) their sequence. *Asterisks* represent stop codons. The entire 3′ ends have not been cloned. However, a putative instability sequence ATTTA is found at the 3′-noncoding region of 75 (*underlined*). Whenever identical, nucleotides or amino acids are shown only in the 75 sequence and represented by a *dashed line* in the 41 sequence. This *dashed line* is interrupted between amino acids 48 and 49 of the 41 sequence by a 50-amino acid insert in the 75 sequence. Amino acids that are identical between this 50-bp insert and a downstream region common to both sequences (amino acids 136–187 of the 75 sequence) are *underlined*. The putative phosphorylation sites for PKA, PKC, and caseine-kinase are *boxed*. The two nuclear targeting motifs are *underlined* in *bold*, and the important amino acids in these motifs are in *boldface capital letters*. *B*, dot matrix analysis of the amino acid sequences of 75 and 41. The dot matrix was performed using the Gene Jockey program with a window of 5. The sequence common to both, which is found repeated with modifications in the 75 sequence, is pointed to by *arrows*.

A

B



(Meurs *et al.*, 1992) was included in the assay as control. Translation was carried out in the presence of 2-aminopurine in order to block any inhibitory effect on the initiation of translation due to activation of the analogous rabbit PKR (Farrell *et al.*, 1977; Katze *et al.*, 1991). The translation (Fig. 4) proved to be at least 10 times more efficient in the presence of 2-aminopurine than in its absence. The recombinant PKR was resolved as a 68-kDa protein, as reported previously (Meurs *et al.*, 1990; Katze *et al.*, 1991). The 41 and 75 RNAs directed the synthesis of 30- and 52-kDa proteins, respectively. While the molecular weight of the 41 protein was in accord with its calculated molecular mass (248 amino acids, 28,569 daltons), the molecular weight of the 75 protein (52 kDa) was higher than expected (371 amino acids, 42,188 daltons). Such a difference was also reported previously for the PKR (68 kDa in gels for a molecular mass of 62 kDa) (Meurs *et al.*, 1990). Differences between the electrophoretic mobility of proteins and their actual molecular mass can be attributed either to post-translational modifications of the translated products or to specific amino acid regions that may bind SDS in an anomalous fashion (Mattaj, 1989). The reason for the decreased electrophoretic mobility of the 75 translated product is not known but may be attributed to a highly localized concentration of basic amino acids brought about by its 50-amino acid insert (10% of the total basic amino acid content). It could also be attributed to a high concentration of cysteine residues (10 cysteines over a 52-amino acid region) in its carboxyl-terminal sequence.

Expression of 75 and 41 cDNAs in Insect Cells Using Baculovirus Vectors—In order to verify *in vivo* some of the properties indicated by sequence data, we analyzed the expression of recombinant 75 and 41 proteins in insect cells using the baculovirus system. Insect SF9 cells were infected either with the wild type baculovirus or with recombinant baculovirus in which the cDNA 75 or 41 have been inserted under control of the polyhedrin promoter. A baculovirus vector in which a catalytically inactive form of PKR cDNA (Barber *et al.*, 1992) has been inserted was included as a control. Three days after infection, cells were pulse-labeled with [^{35}S]methionine, and the total protein extracts were analyzed by SDS-PAGE electrophoresis (Fig. 5*A*). The 32-kDa protein highly expressed in the wild type baculovirus-infected cells represented the viral polyhedrin, the expression of which was abolished in recombinant vectors by cDNA insertion. The recombinant baculovirus PKR migrated as a 68-kDa band, as already reported (Barber *et al.*, 1992). The protein expressed from the recombinant cDNA 75 was found to migrate as a 65–67-kDa band, slightly faster than the PKR. This was again different from its calculated molecular mass, and, more strikingly, it was in contrast with its migration at a 52-kDa protein after translation in the rabbit reticulocyte lysate. This was most probably due to differences in post-translational modifications between the rabbit reticulocyte lysate system and insect cells. It was interesting to note here that an arginine-rich 54-kDa protein was also reported to migrate as a 70-kDa protein in SDS-PAGE (Chaudhary *et al.*, 1991). No protein corresponding to the recombinant 41 could be recovered from insect cells. Instead, the expression of the 41 recombinant baculovirus provoked an accelerated infection pattern, and, although its final virus titers were not affected, it proved to be toxic for the cells (data not shown). The reason for this is not yet known. Further analysis using the baculovirus translation system was then pursued using the 75 recombinant vector alone.

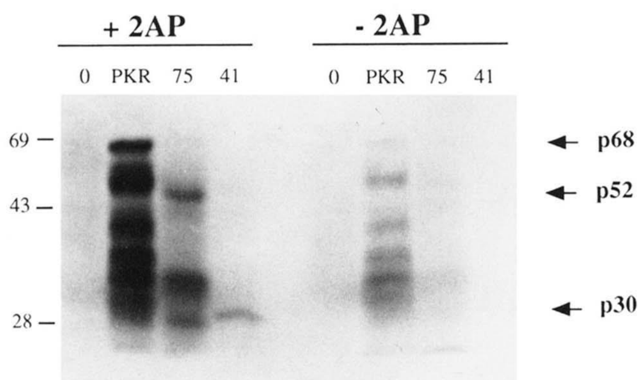


FIG. 4. *In vitro* expression of the 75 and 41 encoded proteins. The 75, 41, and PKR cRNAs, synthesized *in vitro*, from the T7 promoter of pcDNA1/neo vectors, were translated in rabbit reticulocytes lysates (Amersham) in the presence (*+2AP*) or in the absence (*−2AP*) of 2-aminopurine (10 mM) and in the presence of [^{35}S] methionine. The lane marked *0* refers to translation performed in the absence of added RNA. The translated products were separated by SDS-PAGE on a 12.5% acrylamide gels, which were subsequently treated for 30 min with EnHancer (Amersham) and subjected to autoradiography. *Arrows* indicate the position of the PKR protein (p68) and the proteins translated from 75 (p52) and 41 (p30). The smaller sized products in the PKR and 75 lanes probably represent internal initiation or degradation products.

In Vivo Phosphorylation of the 75 Encoded Protein—In view of several potential phosphorylation sites in the 75 protein, we investigated its phosphorylation in insect cells. Three days post-infection, the cells were incubated in presence of [^{32}P]orthophosphate, and the proteins were separated by SDS-PAGE. Results (Fig. 5*B*) show that the 75 protein was indeed phosphorylated *in vivo*. As controls, the PKR was also shown to be phosphorylated, whereas the 40-kDa 2–5A synthetase (Benech *et al.*, 1985) was not phosphorylated. Therefore, *in vivo* phosphorylation of the 75 protein corresponds well with its protein sequence data.

The 75 Protein Is Associated with the Nuclear/Membrane Fraction—The presence of the nuclear targeting motifs in the sequence of 75 indicates that this protein could be nuclear. Insect cells infected with the recombinant 75 baculovirus were either lysed in the presence of SDS to prepare total extracts or in presence of Nonidet P-40 to separate the cytoplasmic and the nuclear/membrane fractions. The results (Fig. 5*C*) show clearly that the 75 protein is associated with the pellet after Nonidet P-40 extraction. In parallel, the wild type baculovirus polyhedrin protein, known to concentrate in the nucleus, was also associated with this pellet. Therefore, the cellular compartmentalization of the 75 protein is in agreement with information of its sequence.

DISCUSSION

We have cloned two new α- and γ-IFN-induced cDNAs, referred to as 75 and 41, which share high homology at the nucleotide and peptide levels. Sequence analysis showed the presence of specific bipartite nuclear target sequences as well as putative phosphorylation sites for several serine/threonine kinases. Both properties were experimentally supported, at least for the 75 protein, since this protein, when expressed in insect cells, was found associated with the nuclear/membrane pellet and could be phosphorylated *in vivo* after expression in recombinant baculovirus-infected insect cells.

The bipartite nuclear target sequences consist of two basic residues followed by a spacer of 10 other residues in which at least 3 out of 5 residues are basic. They are present in many nuclear proteins like nucleoplasmin, steroid hormone receptors, some transcription factors, polymerases, topoisomerases, nucleolin, or p53. These motifs are present at a high percent-

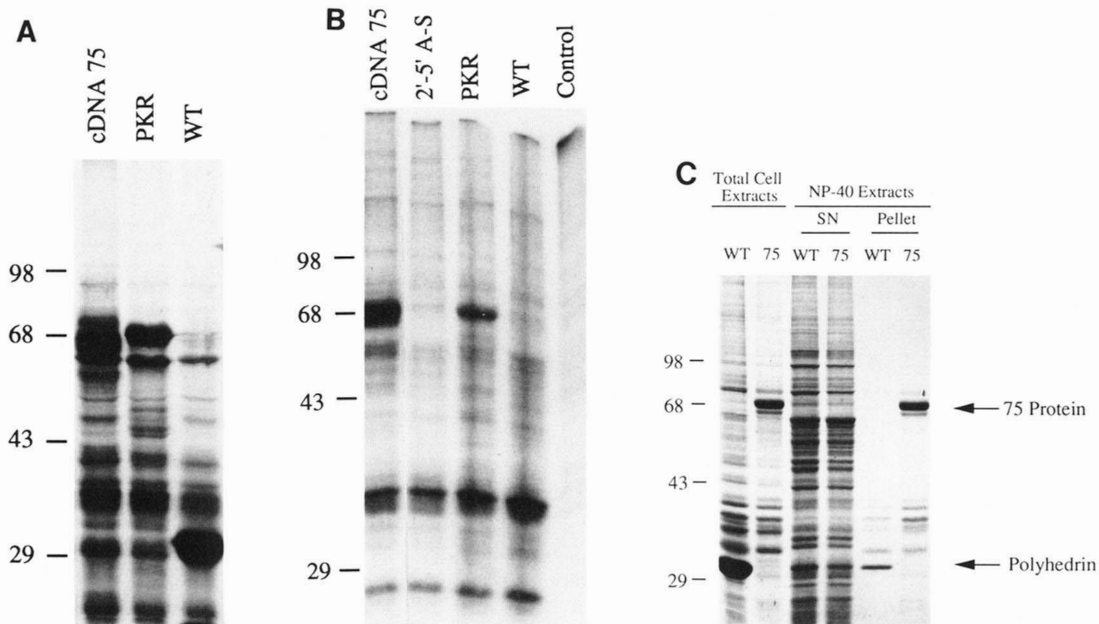


FIG. 5. *A*, *in vivo* expression of the 75 protein using the baculovirus expression vector. Monolayers of insect SF9 cells were infected (10 plaque-forming units/cell) with recombinant baculovirus expressing the cDNA 75. For controls, cells were infected with virus expressing a catalytically inactive form of PKR or with wild-type baculovirus (*WT*). Proteins were labeled with [^{35}S]methionine/cysteine as described under "Experimental Procedures." Total cell lysates were fractionated on 10% polyacrylamide-SDS gels, dried, and autoradiographed. *B*, *in vivo* phosphorylation of the 75 protein in insect cells. Monolayers of insect SF9 cells were either mock-infected (*Control*) or infected with recombinant baculovirus expressing cDNA75, the 2–5A synthetase (*2′–5′A-S*), PKR, and the wild-type baculovirus (*WT*). After 3 days, the monolayers were overlayed with phosphate-free culture medium containing [^{32}P]orthophosphate for 3 h at 28 °C. Whole cell pellets were solubilized and fractionated on 10% polyacrylamide-SDS gels, dried, and autoradiographed. *C*, the 75 protein is associated with nuclear/membrane fractions of cells. Monolayers of insect SF9 cells were infected with either wild-type baculovirus (*WT*) or a recombinant baculovirus expressing the cDNA75 (75). After 3 days of infection, cells were rinsed, collected by centrifugation, and lysed in Nonidet P-40 buffer (see "Experimental Procedures"). Aliquots of cell lysates were denatured in SDS buffer (*Total Cell Extracts*), while the remainder was fractionated by centrifugation at 4000 rpm for 15 min. Nuclear/membrane pellets (Nonidet P-40 Extracts, Pellet) and supernatant fractions (*Nonidet P-40 Extracts, SN*) were denatured in SDS buffer and all samples fractionated on 10% polyacrylamide-SDS gels. Proteins were stained with Coomassie Blue.

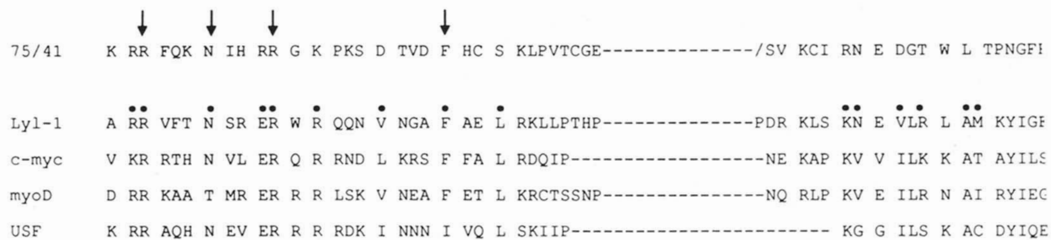


FIG. 6. **Comparison of the 75/41 sequence with some representatives of HLH proteins.** A region of the 75/41 sequence (corresponding to amino acids 199–268 of the 75 sequence, see Fig. 3*A*) presenting homology with the basic region of HLH proteins has been aligned with four members of the HLH family (more than 20 reported to date). These are: Lyl-1 (which is associated with neoplasia of T-ALL), the muscle determination MyoD gene product, the c-*myc* oncogene, and the adenovirus late transcription factor USF. The most highly conserved amino acids are indicated by *dots* above the HLH sequences according to the representation of Visvader and Begley (1991). *Arrows* above the 75/41 sequence point to amino acids that are aligned with the HLH motifs.

age in nuclear proteins and only rarely in the sequence of cytoplasmic proteins (Robbins *et al.*, 1991; Dingwall and Laskey, 1991). The 75 and 41 proteins contain two of these bipartite sequences, one with a spacer of ten amino acids and the other with a spacer of nine amino acids (variation of spacer length was reported not to abolish the targeting efficiency of a protein) (Nath and Nayak, 1990). The existence of two bipartite nuclear target sequences in the 75/41 proteins (whereas most of the nuclear proteins present only one bipartite sequence) is highly suggestive that these proteins migrate to the nucleus.

The similarity of the 75/41 proteins with nuclear proteins was further indicated by the presence of one putative cdc2 phosphorylation site within their first nuclear target sequence (STPKRR) since it is composed of a phosphorylatable residue followed by a proline residue and then by basic residues. For example, other cdc2 substrate motifs found in nuclear proteins are KS/TPK (histones), HSTPPKK (SV40 antigen), and TP*X*KK (nucleolin) (Lewin, 1990). Phosphorylation near nuclear target sequences has been proposed to accelerate the rate of nuclear uptake and to play a role in the cell cycle-dependent control of the nuclear entry of oncogene products and of transcription factors (Robbins *et al.*, 1991).

Based on these theoretical and experimental indications that 75 and 41 could be nuclear proteins, we next examined to which class they might belong. We did not find any structural homology with specific DNA-binding sequences such as zinc-finger, either the C2-H2 TFIIIA model (Miller *et al.*,

1985) or the C2-C2 steroid hormone receptor protein model (Freedman *et al.*, 1988) in spite of the presence of several cysteine residues toward the carboxyl-terminal part of the 75 protein. Neither did we find homeodomains or leucine zipper motifs. We did, however, find some limited homology with HLH motif (Benezra *et al.*, 1990; Blackwood and Eisenman, 1991). This homology was restricted to the basic region preceding helix 1 (4 out of the 5 conserved residues). In the helix 1 region itself, out of 3 conserved residues, only 1 (phenylalanine) was found, and there was no indication of any conserved residue corresponding to the helix 2 region (Fig. 6). Therefore, the only homology shared between 41/75 proteins and some specific DNA-binding proteins comes from the presence in their sequences of an altered version of the basic region typical of HLH proteins. This basic region has been reported to determine some of the DNA-binding specificity (Weintraub *et al.*, 1991)

Our data and sequence analysis suggest that the 41 and 75 are nuclear proteins with some DNA binding properties that are more related to those of histones than to those of some transactivators or replication enzymes. Like histones, they may bind to the minor groove of the DNA using direct amide-base hydrogen bonds and Van de Waals interactions. They could play a structural role or be implicated in the modulation of other factors. For instance, it has been reported that the abundant non-histone chromosomal HMG-1 protein was phosphorylated at metaphase *in vivo*, suggesting a regulation of DNA binding (Lund and Laland, 1990). Interestingly, another high mobility group protein HMGI(Y) was reported recently to bind specifically to PRDII and cooperate in the NFkB induction of the human IFN-β gene (Thanos and Maniatis, 1992). In line with this, it is tempting to speculate that the 75 and 41 proteins, which are interferon-induced, could play a similar role in the regulation of some genes involved in the action of interferon.

The reason for the initial selection of the 1.2 cDNA using polyclonal antibodies raised against the human PKR still remains unexplained. It is possible that these antibodies may have selected an epitope common to the PKR and to the fusion protein expressing the 1.2 coding region. Comparison of the 1.2 and PKR peptide sequences indeed showed a region of good homology composed of six hydrophilic amino acids between 1.2 (SRSKEK, residues 183–188 in 75 sequence) and PKR (SRSKTK) (Meurs *et al.*, 1990). However, neither the *in vitro* translated 75 and 41 proteins nor the *in vivo* expressed 75 protein was recognized by the anti-PKR antibodies in immunoblot analysis. It remains possible that this six-amino acid motif may have been more exposed to the anti-PKR antibody recognition due to partial expression of the 1.2 cDNA instead of the total protein expression. The only information of interest concerning this epitope is that it lies within the protein kinase catalytic subdomains IV and VI of the human PKR and that it also corresponds to one of the potential phosphorylation sites.

Another possibility is that the 75/41 proteins might serve as substrates for the PKR and/or present some binding property toward this enzyme. We showed that the *in vivo* expressed 75 protein could migrate as a 67-kDa protein, very close to the p68 PKR. It is possible, therefore, that trace amounts of it might have purified along with the PKR on the monoclonal antibody-Sepharose used to purify extensively the enzyme and have been subsequently coinjected in mice to raise polyclonal antibodies for the PKR. The only well defined physiological substrate for the PKR is the α subunit of initiation factor eIF2 (Samuel *et al.*, 1984; Chong *et al.*, 1992). We have recently shown that PKR can function as a tumor supressor by a mechanism probably different than eIF2 phosphorylation (Meurs *et al.*, 1993). IkB, the cytoplasmic inhibitor of the transactivation factor NFkB, has been proposed as substrate for the PKR, since it has been shown to be phosphorylated by hemin-regulated inhibitor, the rabbit homolog of PKR (Ghosh and Baltimore, 1990). Whether the 75/41 proteins represent other substrates for the PKR remains to be determined. The preparation of specific antibodies against these proteins is now in progress. Such antibodies are essential for characterization of 75/41 proteins in interferon-treated cells and will help to determine their function.

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