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Molecular Cloning of a New Interferon-induced PML Nuclear Body-associated Protein*

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Transcriptional induction of genes is an essential part of the cellular response to interferons. We have established a cDNA library from human lymphoblastoid Daudi cells treated for 16 h with human /-interferon (IFN) and made use of differential screening to search for as yet unidentified IFN-regulated genes. In the course of this study, we have isolated a human cDNA that codes for a 20-kDa protein sharing striking homology with the product of the Xenopus laevis XPMC2 gene. This new gene is induced by both type I and II IFNs in various cell lines and will be referred to as ISG20 for interferon-stimulated gene product of 20 kDa. Confocal immunofluorescence analysis of the subcellular localization of ISG20 protein reveals that it is closely associated with PML and SP100 gene products within the large nuclear matrix-associated multiprotein complexes termed the PML nuclear bodies.

The interferons (IFNs) are a family of secreted multifunctional proteins that exert a broad spectrum of biological activities. First characterized for their potent antiviral properties, it has now been established that they are involved in a number of regulatory functions such as control of cell proliferation, differentiation, and regulation of the immune system (1, 2). Binding of both type I IFN (IFN- /-) and type II IFN (IFN- /-) to different cell-surface receptors (3, 4) activates transcription pathways via tyrosine phosphorylation of latent cytoplasmic transcription factors termed STAT factors (for signal transducer and activator of transcription) (5–11). The STAT factors are assembled to form the specific transcription complexes ISGF3 (for interferon-stimulated gene factor 3) for IFN- /- and GAF (for IFN- activation factor) for IFN-. These transcription factors act at different cis-acting DNA elements termed the IFN-stimulated responsive element for ISGF3 and the IFN-activation site for GAF and are located in the promoter region of IFN-induced genes (12–18).

The diverse biological actions of IFNs are thought to be mediated by the products of specific but usually overlapping sets of cellular genes induced in the target cells. More recently, some interferon-induced proteins have been located within discrete nuclear structures termed nuclear bodies (19–22). Previously defined by electron microscopy as dense 0.3–0.5-nm diameter spherical particles, the nuclear bodies, so-called PML (for promyelocytic leukemia protein) nuclear bodies (PML NBs), appear characteristic of large multiprotein complexes associated with the nuclear matrix (23–28). These structures are distinct from other well described subnuclear domains such as the nucleolus, the interchromatin granules, the perichromatin fibrils, and the coiled bodies (for review, see Ref. 29). Among the PML NB-associated proteins, PML is the best documented. PML was originally discovered as a fusion protein with retinoic acid receptor- in malignant hematopoietic cells with a t(15;17) translocation characteristic for patients with acute promyelocytic leukemia (reviewed in Refs. 30–32). A tumor cell growth suppressor function and a role in human oncogenesis were reported for PML (33–36). In addition to PML, the nuclear bodies include at least two other IFN-induced proteins, NDP52 (for nuclear dot protein 52) (19) and the SP100 protein, originally identified as an autoantigen in patients with primary biliary cirrhosis (20, 24). It is interesting to note that the number and morphology of PML NBs are variable, particularly throughout the cell cycle and in some pathological contexts. In acute promyelocytic leukemia, PML NBs are disrupted into a microparticulate pattern as a consequence of the expression of the PML-retinoic acid receptor- oncoprotein. Retinoic acid treatment triggers a reorganization of the nucleus to generate normal appearing PML NBs, which in turn is linked to differentiation of acute promyelocytic leukemia cells (26–28).

Although the function of PML NBs is still unknown, some observations suggest that they may represent preferential targets for viral infection and thus could play a role in the mechanism of antiviral action of IFNs. In particular, after adenovirus infection, the viral E4-ORF3 protein is targeted to PML NBs and causes their reorganization from spherical to fibrous structures (37, 38). The human T-cell leukemia virus type 1 Tax oncoprotein induces a diffuse cytoplasmic redistribution of the Int-6 protein, which normally colocalizes with PML in the absence of Tax expression (39). This delocalization appears to be specific for Int-6 because Tax does not alter the global speckled staining pattern of PML. The herpes simplex virus type 1 immediate-early protein Vmw110 (ICPO), which is implicated in the control of reactivation of latent herpes simplex virus type 1, transiently colocalizes after viral infection to PML NBs and subsequently disrupts these structures (40, 41). In the same way, PML NBs are reorganized after human cytomegalovirus infection (42, 43). The Epstein-Barr virus-encoded nuclear antigen EBNA-5 (44), the adenovirus E1A protein, and the SV40 large T antigen (37), other viral members of the
oncoprotein family, are also found in close association with PML NBs. These observations, coupled with the fact that the PML, SP100, and NDP52 proteins are all induced by IFN (19–22), have suggested that PML NBs may play a role in the viral infection process. The elucidation of the actual role of PML NBs in the cell and their implication in the mechanism of action of IFN will be through the identification of new PML NB-associated proteins and the characterization of their behavior under physiological stimuli.

We have therefore established a cDNA library from IFN-treated Daudi cells and made use of differential screening to search for as yet unidentified IFN-regulated genes (45). In the course of this study, we have isolated a human cDNA encoding a novel protein that shares strong similarity with the nuclear Xenopus protein XPMC2 (46). This new gene will be referred to as ISG20 for interferon-stimulated gene product of 20 kDa. Using laser confocal immunofluorescence analysis, we demonstrate that ISG20 protein is closely associated with PML and SP100 in the newly described nuclear structures termed PML NBs.

MATERIALS AND METHODS

Cell Cultures and Antibodies—Human lymphoblastoid Daudi cells were grown in suspension in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum. Hamster fibroblast (CCL39), SV40-transformed monkey kidney epithelial (COS-7m6), human hepatocarcinoma (CCL13), and human HeLa cells were grown in monolayer cultures in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal calf serum. For IFN induction, exponentially growing cells were exposed for 16 h to 500 IU/ml human lymphoblastoid IFN (HuIFN-α; obtained from Hayashibara Biochemical Laboratories Inc.) or 500 units/ml IFN-γ (obtained from Roussel-UCLAF, Paris, France). For the generation of anti-PML and anti-SP100 antibodies, a PML cDNA Novo-Smol fragment (positions 80–1421 in the cDNA sequence) and the full-length coding region of SP100 cDNA were inserted into a gluthathione S-transferase gene fusion vector to generate GST-PML and GST-SP100 protein hybrids. Recombinant proteins produced in Escherichia coli were purified using glutathione-Sepharose 4B columns and were used for rabbit immunizations.

RNA Purification and Northern Blot Analysis—For RNA purification, the cells were pelleted and washed in phosphate-buffer saline, and total mRNAs were isolated by the guanidine thiocyanate method as described previously (47). RNAs were fractionated by electrophoresis on a 10% (v/v) formaldehyde-containing 1.2% (w/v) agarose gel and transferred to nylon membranes (Hybond-N, Amersham Corp.). For the generation of anti-PML and anti-SP100 antibodies, a PML cDNA Novo-Smol fragment (positions 80–1421 in the cDNA sequence) and the full-length coding region of SP100 cDNA were inserted into a gluthathione S-transferase gene fusion vector to generate GST-PML and GST-SP100 protein hybrids. Recombinant proteins produced in Escherichia coli were purified using glutathione-Sepharose 4B columns and were used for rabbit immunizations.

The kinetics of expression of the RNA hybridizing to the ISG20 cDNA probe was analyzed as described above. As shown in Fig. 1A, the ISG20 mRNA accumulated rapidly after the onset of IFN treatment. An 8-fold increase in its steady-state level was reached after 16 h of exposure to IFN. Hybridization of a 2.0-kb cDNA probe used as an invariant control confirmed that each lane of the blot contained an equal amount of total RNA (Fig. 1A). ISG20 participates in the primary response of IFN action, and this induction was not dependent on continuous protein (data not shown).

The specificity of induction of the ISG20 mRNA in response to treatment with the various types of IFN was then analyzed. As Daudi cells failed to respond to HuIFN-α due to the lack of functional receptors, HeLa cells were treated with 500 units/ml HuIFN-α or HuIFN-β, and total RNAs were extracted and analyzed as described above. As shown in Fig. 1B, the kinetics of induction of ISG20 mRNA was found to be similar with the two types of IFN, although HuIFN-β was found to be a stronger inducer.

To determine whether the modulation of ISG20 occurs at the transcriptional level, nuclear run-on assays were conducted.
with nuclei purified from Daudi cells treated or not with HuIFN-β as described previously (48). The labeled RNAs were hybridized with filters containing ISG20, GAPDH, and pBluescript II KS vector probes. GAPDH and the pBluescript II KS vector were used as invariant and negative controls, respectively. The data presented Fig. 1C clearly demonstrate that the increase in ISG20 expression by IFN occurs at the transcriptional level, in keeping with the mechanism of induction of the majority of IFN-induced genes.

To determine the tissue specificity of ISG20 expression, the 694-nucleotide insert was used to probe a set of RNAs isolated from several tissues (multiple-tissue Northern blot membrane from CLONTECH). As shown in Fig. 2, ISG20 is strongly expressed, in the absence of exogenous IFN treatment, in peripheral blood leukocytes, in lymphoid tissues (such as spleen or thymus), and in colon and lung. Various basal levels were detected in other tissues. The same blots were stripped and rehybridized with the control -actin provided by the manufacturer as an invariant control.

Sequence Analysis of ISG20 cDNA—To investigate the function of ISG20, the nucleotide sequence of the 694-nucleotide ISG20 cDNA fragment was determined. A single open reading frame (ORF) of 537 nucleotides was identified (Fig. 3). The ISG20 ORF predicts a gene product (gpISG20) of 179 amino acids with a relative molecular mass of 20.4 kDa. Analysis of the deduced amino acid sequence revealed a very basic protein with 7 lysine and 19 arginine residues (Lys Arg 14.5% of total residues) and a pI of 9.2. Using algorithms to predict the presence of helices (51) and coiled-coil structures (52), region 78–107 of gpISG20 is strongly predicted to form a coiled-coil domain (Fig. 3). This structural domain is known to mediate protein-protein interactions (for review, see Ref. 53), suggesting that ISG20 can act as a component of a multiprotein complex; this important point will be discussed later on the basis of the subcellular localization of ISG20 protein. The coiled-coil region is bordered on both sides by potential phosphorylation sites. A tyrosine kinase phosphorylation site is located from amino acids 44 to 52, and a phosphorylation domain is located from amino acids 106 to 134, which contains several potential protein kinase C, casein kinase II, and cAMP-dependent protein kinase phosphorylation sites (Fig. 3).

A nucleotide comparison by computer search did not reveal any significant homology between ISG20 and the sequences referenced in data bases. However, a search for amino acid sequence homologies revealed that the complete ISG20 protein shares amino acid cluster homologies with the Xenopus laevis XPMC2 gene product (46) and with a theoretical ORF present in the genome of Saccharomyces cerevisiae (GenBank accession number Z74822). Expression of XPMC2 cDNA has been shown to rescue in the fission yeast Schizosaccharomyces pombe several mitotic catastrophe mutants defective in both Wee1 and Mik1 kinases. These redundant kinases negatively regulate Cdc2 kinase by phosphorylating a conserved tyrosine residue. The XPMC2 gene product acts as a negative cell cycle regulator by competing with mitotic substrates for phosphorylation by Cdc2 kinase (46). The role and the regulation of the S. cerevisiae ORF remain unknown.

On the basis of the amino acid homology between ISG20 and XPMC2, whose amino acid sequence alignment is presented in Fig. 4, it is tempting to speculate that ISG20 may be one of the elements that participate in the negative regulation of cell division, which would be consistent with the anti-growth properties of IFNs as found in particular in Daudi cells. We evaluated this hypothesis by analysis of the cell cycle distribution of cells overexpressing ISG20. To this aim, COS-7m6 cells were transiently transfected with a vector expressing ISG20. Transfection was performed by the LipofectAMINE procedure. The cells were collected 72 h later and analyzed with a flow cytometer after propidium iodide staining. Under these experimental conditions, no significant alteration in the cell cycle distribution was observed (data not shown).

The recent release in the data bases of a human 5'-EST

![Fig. 1. Northern analysis of IFN-induced ISG20 mRNA.](image1)

![Fig. 2. Tissue specificity of ISG20 expression.](image2)
sequence (GenBank™ accession number R02224) with a high nucleotide identity to the XPMC2 cDNA clearly demonstrates that ISG20 is not the human homolog of XPMC2, but is a member of a new family of proteins. Surprisingly, human EST-like ISG20 corresponds to a short RNA and encodes a protein homologous only to the carboxylic half of the XPMC2 protein.

Subcellular Localization of ISG20 Protein—The ISG20 amino acid sequence does not enclose a canonic bipartite nuclear localization signal, but the presence of lysine- and arginine-rich domains and the small size of the protein suggest that gpISG20 might be targeted to the nucleus. To determine the subcellular localization of ISG20, CCL13, CCL39, and COS cells were transfected with a tagged ISG20 protein. A fusion cDNA between the open reading frame of ISG20 and the HA epitope peptide sequence of the influenza virus was cloned under the control of the cytomegalovirus promoter in the pJ7 vector (pJ7TagHA-ISG20). Cells were transfected by the calcium phosphate precipitation or LipofectAMINE method and then analyzed by immunofluorescence using the 12CA5 monoclonal antibody. The ectopically expressed ISG20 was predominantly nuclear and gave a speckled distribution pattern in all cell types (Fig. 5A). This localization was not dependent on the level of tagged ISG20 expression since the same pattern was observed in COS cells, which express a high level of transfected cDNA as compared with CCL39 and CCL13 cells. However, in CCL13 cells, ISG20 was diffusely distributed throughout the nucleoplasm in 30% of the positive cells. This percentage strongly suggests that the change in the intranuclear distribution of ISG20 might be dependent on the progression of the cell cycle. The nuclear dots containing ISG20 were dispersed throughout the nucleoplasm and were variable in size and number per nucleus (Fig. 5A). The speckled nuclear staining pattern of overexpressed ISG20 is coincident with that of endogenous PML as shown in Fig. 5B. To examine whether ISG20 and PML exactly localized within the same nuclear structures, confocal immuno-

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**Fig. 3. Nucleotide sequence and predicted amino acid sequence of ISG20 cDNA.** The complete nucleotide sequence of ISG20 cDNA (bottom line) and the predicted amino acid sequence (top line) are shown. The nucleotides and amino acids are numbered to the right of the sequence. The predicted coiled-coil region is boxed. The two phosphorylation domains are underlined.

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**TABLE 2.**

| Name       | Accession Number | Description            |
|------------|------------------|------------------------|
| ISG20      | R02224           | Predicted amino acid   |
|            |                  | sequence of ISG20 cDNA |

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**FIG. 4.** Modulation of PML NB-associated Protein by IFNs

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**FIG. 5.**

A. Nucleotides and amino acids are numbered to the right of the sequence. The predicted coiled-coil region is boxed. The two phosphorylation domains are underlined.

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**FIG. 6.**

B. The punctuate pattern of overexpressed ISG20 is coincident with that of endogenous PML as shown in Fig. 5B. To examine whether ISG20 and PML exactly localized within the same nuclear structures, confocal immu-
nofluorescence microscopic analysis was performed in the CCL13 cells. The localization of the conserved amino acids are indicated by asterisks, and the conserved hydrophobic residues by periods.

Fig. 4. Alignment of amino acid sequences of ISG20 and XPMC2 proteins. The proteins are indicated on the left. The positions of the conserved amino acids are indicated by asterisks, and the conserved hydrophobic residues by periods.

Fig. 5. Subcellular localization of ISG20 protein. A, nuclear localization by immunofluorescence of ectopic HA-ISG20 in transiently transfected CCL39, COS, and CCL13 cell lines; B, localization by double immunofluorescence labeling of ectopic hemagglutinin-ISG20 (right panel) and endogenous human PML (left panel) in CCL13 cells.

Fig. 6. Laser confocal immunofluorescence analysis of colocalization of ISG20 with PML and SP100 in CCL13 cell line. A, localization by double immunofluorescence labeling of ectopic HA-ISG20 (left panel) and endogenous human PML (center panel) in HA-ISG20-transfected CCL13 cells. The single confocal images were superimposed (right panel). B, localization by double immunofluorescence labeling of ectopic HA-ISG20 (left panel) and endogenous human SP100 (center panel) in HA-ISG20-transfected CCL13 cells. The single confocal images were superimposed (right panel).

DISCUSSION

Binding of IFNs to their specific cell-surface receptors triggers the rapid nuclear translocation of a complex formed by association between the various phosphorylated STAT proteins (see the Introduction). This mechanism results in the induction of specific sets of genes that mediate the various biological functions of IFNs. The proteins encoded by these genes exhibit cytoplasmic, nuclear, or cell-surface localization. Cytological analysis has revealed a complex functional organization within the nucleus. Some nuclear proteins localize within discrete and functionally distinct classes of nuclear domains. The two most documented are the nucleolus, in which genes are transcribed and ribosomal RNA is processed (54), and the presumptive
splice sites resulting from the association of small ribonucleoprotein particles with specific sites on the nuclear matrix (55–57). Recently, some IFN-induced proteins have been described firmly bound to the nuclear matrix, forming discrete nuclear structures termed NBs or PML NBs, distinct from the subnuclear domains previously described (19–28). In this report, we describe the isolation and characterization of a cDNA encoding a novel PML NB-associated protein designated ISG20, the expression of which is induced after HulIFN treatment.

Comparison of the amino acid sequence of ISG20 with the sequences of EMBL and GenBank™ Data Banks revealed significant homologies between ISG20 and the X. laevis XPMC2 gene product (46). Using a genetic complementation method, XPMC2 has been identified to functionally rescue a fission yeast mitotic catastrophe mutant defective in both Weel and Mki1 kinases (46). These homologies raise the possibility that ISG20 might act as a negative regulator of cell division induced by IFNs. However, ISG20 did not appear to affect cell growth in transient transfection experiments. Alignment of the amino acid sequences of ISG20 and XPMC2 showed that ISG20 is much shorter than XPMC2 and is homologous to the C-terminal half of XPMC2 protein. Interestingly, a truncated XPMC2 protein that retains only its C-terminal half is not able to rescue the mitotic catastrophe phenotype. According to the presence within ISG20 protein, of a presumptive structural coiled-coil domain that is supposed to mediate protein-protein interactions, we can imagine that interactions between ISG20 and other proteins are required to mimic, in human, the XPMC2 function. However, we have no direct evidence to credit this hypothesis at present. In addition, ISG20 shares a strong amino acid homology with a theoretical ORF present in the genome of S. cerevisiae. The function of this ORF remains unknown. Since protein conservation during evolution usually affects essential cellular functions, the comprehension of the regulation and biological activity of the S. cerevisiae ORF will be important to determine the actual role of ISG20. The knock-out of the yeast gene is now in progress to allow this study.

We have examined the subcellular localization of ISG20 protein. Ectopic expression of a fusion protein between ISG20 and the HA epitope revealed that ISG20 is predominantly nuclear and gives a punctuate staining pattern. Interestingly, the number and size of the nuclear dots containing ISG20 were variable from one cell to another. These data can reflect a modulation of PML NBs throughout the cell cycle. Using confocal immunofluorescence microscopy, we demonstrated that ISG20 is closely associated with PML and SP100 within the large multiprotein complexes termed PML NBs (19–28). To determine whether a direct interaction between these proteins can occur, a yeast two-hybrid study was performed. This was conducted using ISG20 fused to the GAL4 DNA-binding domain and PML or SP100 fused to the GAL4-activating domain. No direct interaction was detected between these proteins when coexpressed in yeast Y187 cells.

Viruses require the host cell machinery for their multiplication cycle, and they have developed various strategies to circumvent the antiviral activities induced in the cells by IFNs. Various viral proteins such as the adenovirus E4-ORF3 protein (37, 38), the human T-cell leukemia virus type 1 Tax oncoprotein (39), the herpes simplex virus type 1 Vmw110 protein (40, 41), the cytomegalovirus promoter IE1 protein (43), and the SV40 large T antigen (37) concentrate in the PML NB speckles, suggesting that PML NBs play a major role during infection by oncogenic viruses. The fact that the PML NB-associated proteins PML, SP100, NDP52, and ISG20 are all inducible by IFNs (Refs. 19–22 and this report) strongly suggests the implication of a such subnuclear structure in the mechanism of IFN action. Further studies on the modulation of Int-6 and PIC-1, a newly described PML NB-associated protein (58, 59), by IFNs might be worth being performed to confirm this hypothesis. The disruption of PML NBs during viral infection reflects the ability of viruses to circumvent the antiviral activities of IFNs. Eventual modifications of ISG20 subcellular localization after various viral infections are underway to increase the understanding of the role of ISG20 in the mechanism of antiviral action induced by IFNs. The availability of specific antibodies against this protein is essential to determine its function, and their preparation is now in progress.

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