

Interferon Action: Nucleolar and Nucleoplasmic Localization of the Interferon-inducible 72-kD Protein That Is Encoded by the Ifi 204 Gene from the Gene 200 Cluster

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Abstract. The interferons are cytokines with antiviral, cell growth regulatory, and immunomodulatory activities. These activities are mediated by the proteins induced by the interferons. Earlier we described a gene cluster (the 200 cluster) consisting of at least six adjacent, interferon-activatable genes located next to the erythroid alpha-spectrin locus on murine chromosome 1. The genes of the cluster arose by repeated gene duplication and they specify proteins with pronounced sequence similarity. We have now raised polyclonal antibodies against a segment from one of these proteins (the 204 protein of 72 kD). Using these, we established that the 204 protein is a phosphoprotein whose level in cells from various murine lines can be increased up to 75-fold upon treatment with alpha in-

terferon. Experiments involving fractionation of cell lysates and indirect immunofluorescence microscopy of control and interferon-treated cells revealed that the 204 protein is nucleolar and nucleoplasmic. This conclusion was confirmed by co-localization with B23, a known nucleolar protein. The 204 protein is the first interferon-induced protein found to be located in the nucleoli, the subcellular organelles of ribosomal RNA production and ribosome assembly. It remains to be seen whether the 204 protein affects any of these processes. Studies on 204 protein function should be facilitated by the availability of complete cDNA clones and the finding of cell lines in which the expression of this protein is impaired.

THE interferons (IFNs)¹ are a family of cytokines with antiviral, cell growth regulatory, and immunomodulatory activities (Lengyel, 1982; Pestka et al., 1987; De Maeyer and De Maeyer-Guignard, 1988). There are three classes of IFNs: alpha (in humans—at least 15 species), beta (in humans—one species), and gamma (in humans—one species). The binding of IFNs to specific cell surface receptors on cells from vertebrates induces the synthesis of a large variety of proteins. The alpha and beta IFNs on the one hand and gamma IFN on the other hand induce distinct but overlapping sets of proteins. The induction appears to be primarily the consequence of an enhanced rate of transcription of particular genes. The inducible proteins appear to mediate various activities of the IFNs. Thus, e.g., particular inducible proteins from the Mx family impair the replication of the influenza and vesicular stomatitis viruses (Staheli et al., 1986; Pavlovic and Staheli, 1991), an inducible (2'-5') oligoadenylate synthetase isoenzyme blocks the replication of mengo and encephalomyocarditis viruses (Chebath et al., 1987; Rysiecki et al., 1989), the inducible indolamine 2,3 dioxygenase inhibits the multiplication of the protozoon *Toxoplasma gondii* (Pfefferkorn, 1984) and inducible major histo-

compatibility complex proteins are involved in the functioning of the immune system in various ways (Srivastava et al., 1991).

The subcellular locations of several IFN-induced proteins have been determined. Some proteins from the Mx family were localized in the nucleus and others in the cytoplasm (Pavlovic and Staheli, 1991). Some isoenzymes from the (2'-5') oligoadenylate synthetase family were localized in the nucleus; others in the cytoplasm or associated with membranes (St. Laurent et al., 1983; Chebath et al., 1987). In the case of some IFN-induced proteins with antiviral activity, the proteins may be located at the sites of replication of the viruses they control (Staheli, 1990; Pavlovic and Staheli, 1991).

We established earlier the cDNA sequence encoding an IFN-inducible 72-kD murine protein (the 204 protein) (Choubey et al., 1989). This protein includes two apparently contiguous, ~200-amino acid-long segments with much (34%) sequence similarity, as well as a region including four perfect and three imperfect repeats of a seven-amino acid-long segment. The 204 protein is encoded by the Ifi 204 gene which is part of a cluster (the 200 cluster) of at least six IFN-inducible murine genes (Opdenakker et al., 1989; Kingsmore et al., 1989). The cluster was mapped to murine chromosome 1, between the erythroid alpha-spectrin and the amyloid P-component loci. The mRNAs specified by the

1. Abbreviation used in this paper: IFN, interferon.

genes from the cluster share much sequence similarity and the cluster appears to have evolved by repeated gene duplication (Choubey et al., 1989). The functions of the proteins specified by the gene cluster remain to be established.

Here, we report the first step towards this goal—the preparation and use of a polyclonal antiserum which specifically recognizes the 204 protein. Our studies reveal that the 204 protein is IFN inducible and is phosphorylated. Its IFN-induced level is different in different inbred strains of mice. Moreover, the 204 protein is located in the nucleolus and nucleoplasm of interphase murine cells.

Materials and Methods

Reagents

Recombinant human IFN alpha 2/alpha 1-83 (sp act 8.1×10^7 U/mg) was a generous gift from H. Weber and C. Weissmann (University of Zurich, Zurich, Switzerland). This human IFN was shown to be highly active in murine cells (Weber et al., 1987).

Cell Lines and Growth Conditions

Mouse AKR 2B (obtained from H. Moses, Vanderbilt University, Nashville, TN; Getz et al., 1976), BALB/c 3T3 (clone A31) (ATCC CCL 163; obtained from C. Stiles, Dana Farber Cancer Institute, Boston, MA), BLK SV HD.2 A.5R.1 VA.3R.1 (BLKS) (ATCC TIB 88), and L929 (ATCC CCL 1) cells were grown in monolayers in DME medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% FBS. NIH 3T3 (obtained from R. Weinberg, The Whitehead Institute, Cambridge, MA) (ATCC CRL 1658) and Ehrlich ascites tumor cells (obtained from E. Henshaw, University of Rochester, Rochester, NY) were grown in DME medium, supplemented with 5% calf serum. The cells were passaged when reaching semi-confluency (40–60% confluency). If so indicated, IFN was added (unless otherwise stated, to semiconfluent cultures and at 1,000 U/ml) for 24 h. For labeling with [35 S]methionine, cells were grown in methionine minus DME medium, supplemented with 5% dialyzed calf serum in the presence of IFN for 1 h. Thereafter, the same medium was supplemented with [35 S]methionine (200 μ Ci/ml) (sp act 1,000 Ci/mmol; Amersham Corp., Arlington Heights, IL) together with IFN and the incubation was continued for three more hours. For 32 P-labeling, the cells were grown in DME medium in the presence of IFN for 10 h and subsequently in phosphate-free DME medium (Gibco Laboratories) supplemented with 32 P-orthophosphate (100 μ Ci/ml; Amersham Corp., Arlington Heights, IL) in the presence of IFN, overnight. The control cultures were not treated with IFN.

Preparation of a 204-fusion Protein

A polyclonal antiserum was raised in a rabbit against the NH₂-terminal segment of the 204 protein: the cDNA fragment from nucleotide 89 to 742 (see Fig. 2 in Choubey et al., 1989), (Ball-BanII fragment) coding for this polypeptide was blunt end ligated in the SmaI site of the pGEX-1 *Escherichia coli* expression vector (Amrad Corporation, Kew, Victoria, Australia) to regenerate the proper reading frame. The 5' cloning site of the 204 cDNA insert was confirmed by sequencing. The glutathione transferase-204 fusion protein was expressed in *E. coli* XL-1 blue and was affinity purified on glutathione-agarose beads (Sigma Chemical Company, St. Louis, MO) according to the method of Smith et al. (1988).

Preparation and Immunoaffinity Purification of Antibodies to the 204-fusion Protein

The affinity purified fusion protein preparation was subjected to SDS-PAGE at the preparative scale and the major Coomassie blue-stainable band was cut out and used for immunization of a rabbit at the Pocono Rabbit Farm and Laboratory (Dutch Hill Road, Canadensis, PA) according to their procedure. The antibodies to the 204 protein were immunoaffinity purified by passing the antiserum through a column in which the antigen had been coupled to tresyl-activated agarose (American Bioanalytical, Schleicher and Schuell, Keene, NH) as suggested by the supplier. The antibodies eluted from the column at alkaline pH (11.5) were immediately neutralized and were stored at -20°C .

Subcellular Localization of the 204 Protein: Fractionation of Lysates from AKR 2B Cells

(See Fig. 5) The procedure of Yung et al. (1985) was followed for lysing the cells in a hypotonic buffer, fractionating the lysate into a cytoplasmic and nuclear fraction and subfractionating the nuclear fraction into nucleoplasmic and nucleolar subfractions. The nuclear and nucleolar pellets were extracted with RIPA buffer and 50- μ g protein aliquots from these two extracts and from the cytoplasmic and nucleoplasmic fractions were assayed for protein 204 content by immunoblotting with the anti-204 antiserum.

Assay of the Binding of the 204 Protein in Isolated Nuclei: Treatment with Various Concentrations of NaCl

The lysate from IFN-treated AKR 2B cells was prepared and the nuclei were isolated from the lysate according to the procedure of Ohtsubo et al. (1989). The nuclei were incubated with NaCl at the concentration indicated in 10 mM Hepes (pH 7.5), 5 mM KCl, and 2 mM MgCl₂. Thereafter, the reaction mixtures were centrifuged at 1,000 g for 5 min and equal aliquots from the supernatant fractions (extract) and the pellet fractions (residual nuclei) were boiled in Laemmli's sample buffer (Laemmli, 1970) before assaying for 204 protein by immunoblotting.

Immunoprecipitation and Immunoblotting

Unless otherwise indicated, cells were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris (pH 8.0) supplemented with 1 mM PMSF and 50 μ g/ml leupeptin), briefly sonicated and the cell debris was pelleted. The supernatant fraction was collected and used for protein estimation with the Bio-Rad kit (Bio-Rad Laboratories, Cambridge, MA). Immunoprecipitations were performed using the appropriate dilutions of the preimmune serum or of the anti-204 antiserum (1,000–1,200-fold dilution) or of the immuno-affinity-purified anti-204 antibodies (50-fold dilution) in RIPA buffer at 4°C overnight. The precipitate was incubated with protein A-Sepharose beads (Sigma Chemical Co.), 30–50 μ l in a 50% suspension in RIPA buffer at 4°C for 90 min, collected by centrifugation, and washed with RIPA buffer, three to five times. Finally, the beads were boiled in the sample loading buffer of Laemmli (1970) for 4–6 min and pelleted by centrifugation. The supernatant fractions were subjected to SDS-PAGE. The gel was soaked in an enhancer (ResolutionTM; E. M. Corporation, Chestnut Hill, MA) and processed for fluorography. For immunoblotting, the proteins were transferred from the polyacrylamide gels to Immobilon-P (Millipore Corporation, Bedford, MA) as described (Harlow et al., 1988) and were visualized by staining with a protein stain poncseau S (Sigma Chemical Co.). After blocking with 5% nonfat dry milk in TBS (50 mM [Tris, pH 7.5], 300 mM NaCl) the blot was immunostained with the anti-204 antibodies (diluted 1,200-fold) at 4°C overnight. The antibodies were detected using affinity purified ^{125}I -protein A (30 mCi/mg) (Amersham Corp.), and autoradiography. Unless otherwise indicated, the unpurified anti 204-antiserum was used for immunoprecipitations and immunoblotting.

Indirect Immunofluorescence Microscopy

This was performed on IFN-treated and control AKR 2B cells that were grown on cover slips, washed with PBS (pH 7.2), fixed with freshly diluted 2% formaldehyde in PBS at room temperature for 20 min and permeabilized with (-20°C) methanol at room temperature for 4 min or with 0.1% Triton X-100 in PBS at room temperature for 5 min. After blocking with 30% FBS in PBS at 37°C for 1 h, the cover slips were incubated with pre-immune serum (diluted 500-fold) or immunoaffinity purified 204 antibodies (diluted eightfold) or anti-B23 mAbs (diluted 30-fold) in 3% BSA in PBS at 4°C for 2 h. The cover slips were then washed three times for 5 min each time with PBS.

For staining of the 204 protein the cells were incubated with secondary, fluorescein-labeled sheep, anti-rabbit IgG antibodies (Cappel Laboratories, Durham, NC) at the appropriate dilution in 3% BSA in PBS at 37°C for 30 min. For staining of the B23 protein, the cells were incubated with secondary, rhodamine-labeled goat anti-mouse IgG antibodies (Sigma Chemical Co.) at a 30-fold dilution in PBS at 37°C for 30 min. In the case of double immunofluorescence experiments, the two primary antibodies (i.e., the anti-204 and the anti-B23 antibody) were incubated together as

was the pair of secondary antibodies. The cells were mounted in 3% propyl gallate in 70% glycerol and viewed under the microscope.

In Vitro Transcription and Translation

The 204-cDNA insert in pBluescript (Stratagene, La Jolla, CA) was transcribed using T7 RNA polymerase, and the mRNA obtained was translated in a rabbit reticulocyte lysate supplemented with [³⁵S]methionine as described by Choubey et al. (1989).

Partial Proteolysis

(Fig. 1 c). The 204 protein labeled with [³⁵S]methionine, was immunoprecipitated from IFN-treated AKR 2B cells and subjected to SDS-PAGE, together with the 204 protein translated in vitro. The gel was dried without fixation of the proteins and autoradiography was performed. A slice from the gel with the 204 protein band was cut out from the dried gel, inserted into a second SDS-polyacrylamide gel, subjected to limited digestion with *S. aureus* V8 protease (Boehringer-Mannheim Biochemicals, Indianapolis, IN) and SDS-PAGE was performed as described by Cleveland et al. (1983).

Results

The Anti-204 Antiserum Specifically Recognizes an IFN-induced Murine Protein whose Mobility and Peptide Map Are Indistinguishable from Those of an In Vitro-translated 204 Protein

A polyclonal antiserum was generated in a rabbit injected with a fusion protein, including a 204 protein segment of over two hundred amino acids. The unpurified antiserum

was immunoaffinity purified by binding to and elution from the insolubilized fusion protein. The antiserum turned out to be suitable for both immunoblotting (Fig. 1 a) and immunoprecipitation (Fig. 1 b). In immunoblotting, the unpurified antiserum recognized in a total cell extract from AKR 2B cells a 72-kD protein (Fig. 1 a, lanes 1 and 2) which was not recognized by the preimmune serum (lanes 3 and 4). Treatment of a semiconfluent culture of cells with IFN for 24 h increased the level of the 72-kD protein >50-fold (Fig. 1 a, compare lanes 1 and 2). In the case of immunoprecipitation, the anti-204 antiserum (Fig. 1 b, lane 4) and also the immunoaffinity purified anti-204 antibodies (lane 6) precipitated an IFN-induced 72-kD protein which was not precipitated by the pre-immune serum (Fig. 1 b, lane 2). The level of the 72-kD protein was greatly increased by the IFN treatment (Fig. 1 b, compare lanes 4 to 3 and 6 to 5). The mobility of the 72-kD protein was indistinguishable from that of a 204 protein translated *in vitro* (the band with the lowest mobility in Fig. 1 b, lane IVT). (It should be noted that in the lane IVT, the band with the lowest mobility corresponds to the 204 protein whose translation started at the 5' proximal AUG initiator codon of the 204 mRNA. The heavier band with the next lowest mobility results from starting the translation of the mRNA at the second AUG codon from the 5' terminus) (Choubey et al., 1989). The apparent comigration of the *in vivo*-synthesized 204 protein with the *in vitro*-translated 204 protein indicates that the protein *in vivo* does not undergo posttranslational processing resulting in a

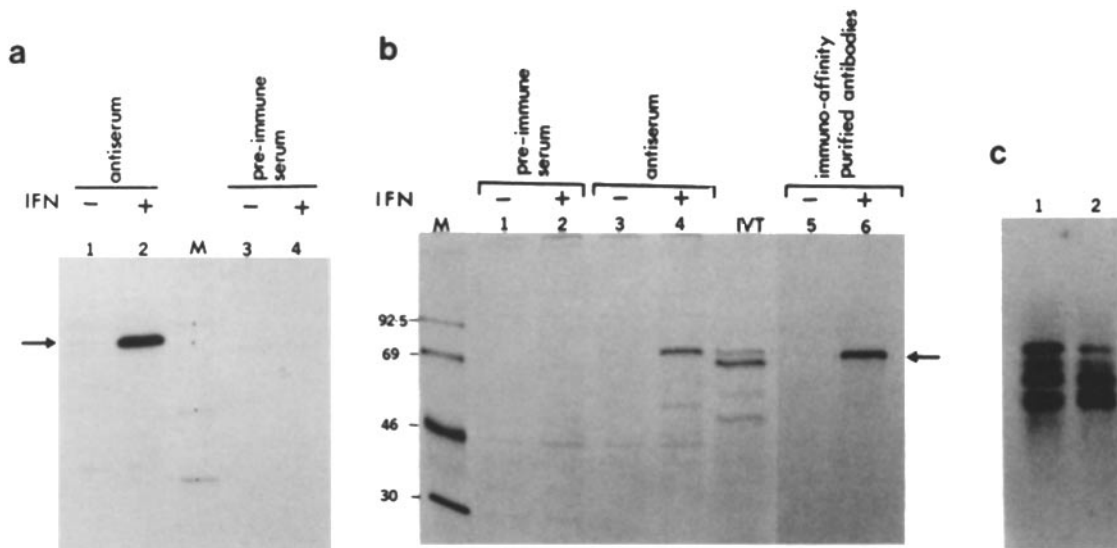


Figure 1. Identification of the IFN-inducible 204 protein using the anti-204 polyclonal antiserum. (a) Immunoblotting. Total cell extracts (100 μ g protein per lane) from control (lanes 1 and 3) or IFN-treated (lanes 2 and 4) AKR 2B cell cultures were fractionated by SDS-PAGE and transferred to Immobilon-P membranes. These were immunostained with the anti-204 antiserum (lanes 1 and 2) or the preimmune serum (lanes 3 and 4), and ¹²⁵I protein A followed by autoradiography. The ¹⁴C-labeled molecular size markers used (in lane M) were phosphorylase B, 92.5 kD, BSA 69 kD, and ovalbumin 46 kD (Amersham Corp., Arlington Heights, IL). The 204 protein band is indicated by an arrow. (b) Immunoprecipitation of [³⁵S]methionine-labeled protein. Total cell extracts from [³⁵S]methionine-labeled control (lanes 1, 3, and 5) or IFN-treated AKR 2B cells (lanes 2, 4, and 6) were immunoprecipitated with the preimmune serum (lanes 1 and 2), the anti-204 antiserum (lanes 3 and 4) or immunoaffinity purified, anti-204 antibodies (lanes 5 and 6). The immunoprecipitates were subjected to SDS-PAGE and visualized by fluorography. [³⁵S]methionine-labeled 204 protein was prepared by translating an *in vitro*-synthesized 204 transcript in a rabbit reticulocyte lysate and was analyzed by SDS-PAGE and fluorography (lane IVT). The molecular size markers used (in lane M) were, as in a, except that carbonic anhydrase 30 kD was also included. The 204 protein band is indicated by an arrow. (c) Limited proteolysis of the 204 protein. Aliquots of the [³⁵S]methionine-labeled 204 protein, immunoprecipitated from IFN-treated AKR 2B cells (lane 1) or translated *in vitro* (lane 2), were subjected to limited proteolysis with *S. aureus* V8 protease in SDS-polyacrylamide gels and the digests were analyzed by SDS-PAGE. For further details, see Materials and Methods.

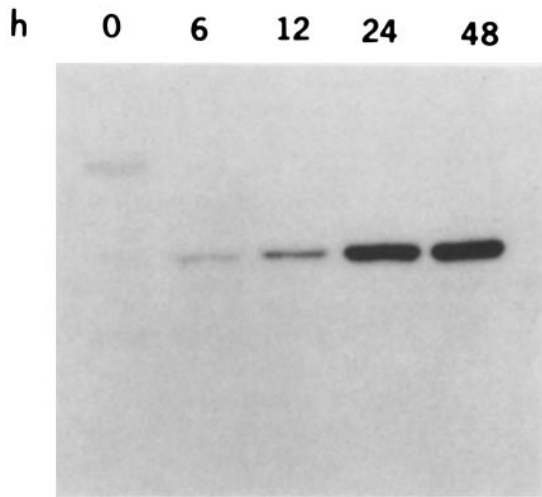


Figure 2. Time course of the induction of the 204 protein by alpha IFN in cultured AKR 2B cells. Semiconfluent cultures were treated with IFN for the time (in hours) indicated. Total extracts (100 μ g protein per lane) prepared from the cultures were fractionated by SDS-PAGE and analyzed by immunoblotting with the anti-204 antiserum. For further details see Materials and Methods.

change in mobility detectable by SDS-PAGE as used. The identity of the *in vivo*-synthesized and *in vitro*-translated 204 proteins was further supported by the outcome of peptide mapping: [35 S]methionine-labeled 204 protein was synthesized *in vivo* and immunoprecipitated. The immunoprecipitate was purified by SDS-PAGE and subjected to partial digestion with *S. aureus* V8 protease, together with [35 S]methionine-labeled 204 protein translated *in vitro*. The peptides generated from the two proteins revealed identical patterns of mobility on SDS-PAGE (compare lanes 1 and 2 in Fig. 1 c).

Time Course of the Induction of the 204 Protein by IFN in AKR 2B Cells

The level of 204 protein in AKR 2B cells not treated with IFN was low but detectable by immunoblotting (Fig. 2). Exposure of a semiconfluent culture to IFN resulted in a pronounced increase in this level after 6 h. After a 24-h exposure, the increase rose to \sim 50-fold and after 48 h to about 75-fold (Fig. 2).

The half-life of the 204 protein was >9 h (as determined in cells which had been exposed to IFN for 24 h before the addition of the protein synthesis inhibitor cycloheximide; 50 μ g/ml; Sigma Chemical Co.) (not shown).

The 204 Protein Can Be Phosphorylated *In Vivo*

The inspection of the amino acid sequence of the 204 protein (Choubey et al., 1989) revealed the occurrence of potential sites for phosphorylation by various protein kinases (Kemp and Pearson, 1990). This prompted us to test whether the 204 protein was phosphorylated *in vivo*. For this purpose, control and IFN-treated cultures of AKR 2B cells were labeled by incubation with 32 P-orthophosphate and aliquots containing equal numbers of 32 P cpm were immunoprecipitated with the anti-204 antiserum and subjected to SDS-PAGE and autoradiography. The presence of 32 P-labeling in

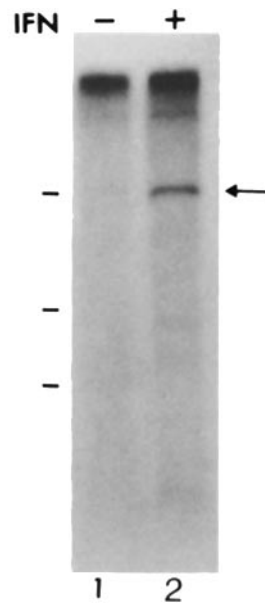


Figure 3. The 204 protein is phosphorylated. Immunoprecipitation of 32 P-labeled 204 protein from control and IFN-treated AKR 2B cells. Total cell extract (100 μ g protein per lane with equal numbers of 32 P-cpm) from a 32 P-labeled control culture (lane 1) and a 32 P-labeled culture treated with IFN (lane 2) were immunoprecipitated with the anti-204 antiserum and subjected to SDS-PAGE and autoradiography. Horizontal lines indicate the bands corresponding to 14 C-labeled molecular size markers (in order of increasing mobility: BSA 68 kD, ovalbumin 46 kD, and carbonic anhydrase 30 kD). The 204 protein band is indicated by an arrow. For further details see Materials and Methods.

the immunoprecipitated 72-kD protein in IFN-treated cells indicated that the 204 protein was phosphorylated in IFN-treated cells (in Fig. 3). No phosphorylation of 204 protein was detected in control cells. However, this undetectability might be the consequence of the very low level of 204 protein in control cells (only 1/50 of that in IFN-treated cells). Thus our results are no basis for conclusions concerning the phosphorylation of the 204 protein in control cells.

Differences in the Inducibility of the 204 Protein by IFN in Cell Lines Derived from Various Inbred Strains of Mice

Studies with mRNAs from various inbred strains of mice and from cell lines derived from the strains uncovered differences in the inducibility of the mRNAs from the gene 200 cluster (Toniato, E., and P. Lengyel, unpublished data; Gariglio et al., 1992). Immunoblotting involving the use of the anti-204 antiserum also revealed such differences among murine cell lines. Thus, the 204 protein was inducible by IFN in cultures of BALB/c 3T3 (Fig. 4, lanes 5 and 6), NIH 3T3, L929, and EAT cells (not shown) to a similar extent as in cultures of AKR 2B cells (Fig. 4, lanes 1 and 2). However, in BLKS cells (a line derived from the C57 BL/6 inbred strain of mice), the constitutive level of the 204 protein was not detectable and IFN treatment increased the level only to that present in control AKR 2B cells.

The BLKS cell line in which the inducibility of the 204 protein by IFN is so much poorer than in many other cell lines might be of use in future studies on the function of the 204 protein.

Subcellular Localization of the 204 Protein: Fractionation of Lysates from AKR 2B Cells

To determine the subcellular location of the 204 protein, cultures of control and IFN-treated AKR 2B cells were lysed, the lysate was fractionated, and the fractions obtained were

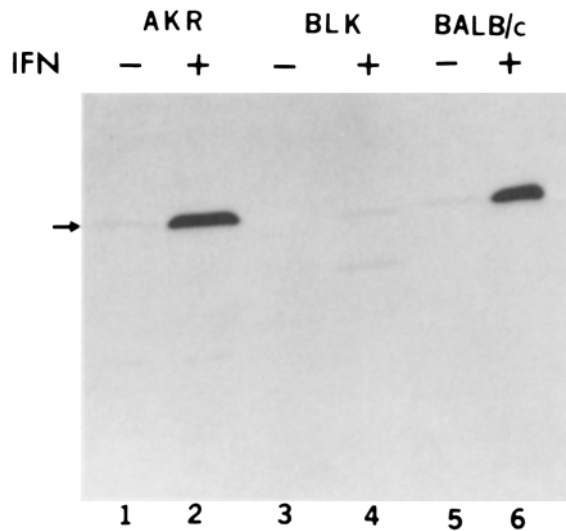


Figure 4. Differences in the inducibility of the 204 protein by IFN in fibroblast lines derived from various inbred strains of mice. Total cell extracts (100 μ g protein per lane) from control cultures (lanes 1, 3, and 5) or cultures exposed to 1,000 U/ml of IFN for 24 h (lanes 2, 4, and 6) from AKR 2B cells (*AKR*; lanes 1 and 2) BLKS cells (*BLK*; lanes 3 and 4) and BALB/c 3T3 cells (*BALB/c*; lanes 5 and 6) were fractionated by SDS-PAGE and analyzed by immunoblotting with the anti-204 antiserum. For further details see Materials and Methods.

assayed for 204 protein by SDS-PAGE and immunoblotting. The patterns in Fig. 5 reveal that in the case of IFN-treated cells, the bulk of 204 protein (>98%) was nuclear (Fig. 5, lane 6), only <2% was cytoplasmic (Fig. 5, lane 5). Furthermore, within the nuclear fraction, 40% of the 204 protein was nucleolar (Fig. 5, lane 8) and 60% nucleoplasmic (Fig. 5, lanes 7). In the case of the lysate from control cells, little 204 protein was detectable in this experiment (Fig. 5, lanes 1-4).

Nucleolar and Nucleoplasmic Localization of the 204 Protein: Indirect Immunofluorescence Microscopy of Control and IFN-treated AKR 2B Cells Stained with Anti-204 Antibodies and Anti-B23 Antibodies: Effect of Actinomycin D Treatment

For immunofluorescence microscopy the AKR 2B cells were grown, treated with IFN (if so indicated) on cover slips, fixed, permeabilized, stained with preimmune serum or immunoaffinity purified rabbit anti-204 antibodies, and visualized with fluoresceine-labeled sheep anti-rabbit IgG. The use of preimmune serum resulted in no detectable staining of cells (not shown). The photomicrographs in Fig. 6, *a* and *b* reveal that the immunoaffinity purified anti-204 antibodies gave rise to a very faint staining of the nuclei in control cells and a bright staining of the nuclei in IFN-treated cells. The cytoplasm was very poorly stained, even in the case of IFN-treated cells.

The pattern of nuclear staining in both IFN-treated and control cells was punctate and characteristic of interphase nucleoli in these cells. In cells treated with IFN, the nucleoplasm was also stained (although much less brightly than the nucleoli). Furthermore, the brightness of nuclei in different IFN-treated cells was not quite uniform.

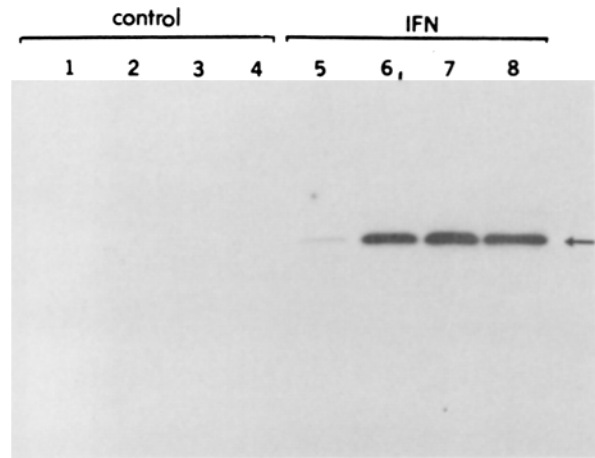


Figure 5. Subcellular localization of the 204 protein: fractionation of lysates from AKR 2B cells. Extracts from control (lanes 1 to 4) or IFN-treated cells (lanes 5 to 8) were fractionated into cytoplasmic (lanes 1 and 5) and nuclear fractions (lanes 2 and 6). The nuclear fraction was further subfractionated into nucleoplasmic (lanes 3 and 7) and nucleolar fractions (lanes 4 and 8). Aliquots (containing 50- μ g protein from each fraction) were tested for 204 protein by immunoblotting with the anti-204 antiserum. The 204 protein band is indicated by an arrow. For further details see Materials and Methods.

To verify the nucleolar localization of the 204 protein a mAb against the nucleolar B23 protein (Lischwe et al., 1979; Yung et al., 1990) was used. After fixation and permeabilization of IFN-treated cells, they were first stained with both the immunoaffinity purified anti-204 antibodies and the monoclonal anti-B23 antibodies and the two antibodies were visualized (in the case of the anti-204 antibodies with fluoresceine-labeled anti-rabbit IgG antibodies and, in the case of anti-B23 antibodies, with rhodamine-labeled anti-mouse IgG antibodies). The patterns in Fig. 7 reveal that the 204 protein is located in the same subcellular organelles (*a*) as the nucleolar B23 protein (*b*). This colocalization of the 204 protein with the nucleolar B23 protein clearly indicates that much of the 204 protein is located in the nucleoli in interphase AKR 2B cells.

As a further test of the localization of the 204 protein, we tested the effect of actinomycin D treatment of cells on the immunofluorescence staining pattern. Actinomycin D, an agent intercalating into DNA, is a potent inhibitor of transcription and electron microscopic studies revealed that the exposure of cells to this agent results, among other changes, in nucleolar segregation, i.e., the dispersion of some nucleolar components (Busch and Smetana, 1970).

In line with the nucleolar localization of the 204 protein, we found that the exposure of IFN-treated cells to actinomycin D (1 μ g/ml for 1 h) changed the immunofluorescence pattern of the 204 protein from punctate and characteristic of nucleoli to a more dispersed, nucleoplasmic pattern (Fig. 8, *a* and *b*).

Characteristics of the Binding of the 204 Protein in Isolated Nuclei

Nuclei (isolated from IFN-treated AKR 2B cells) were incubated with increasing concentrations of NaCl and the

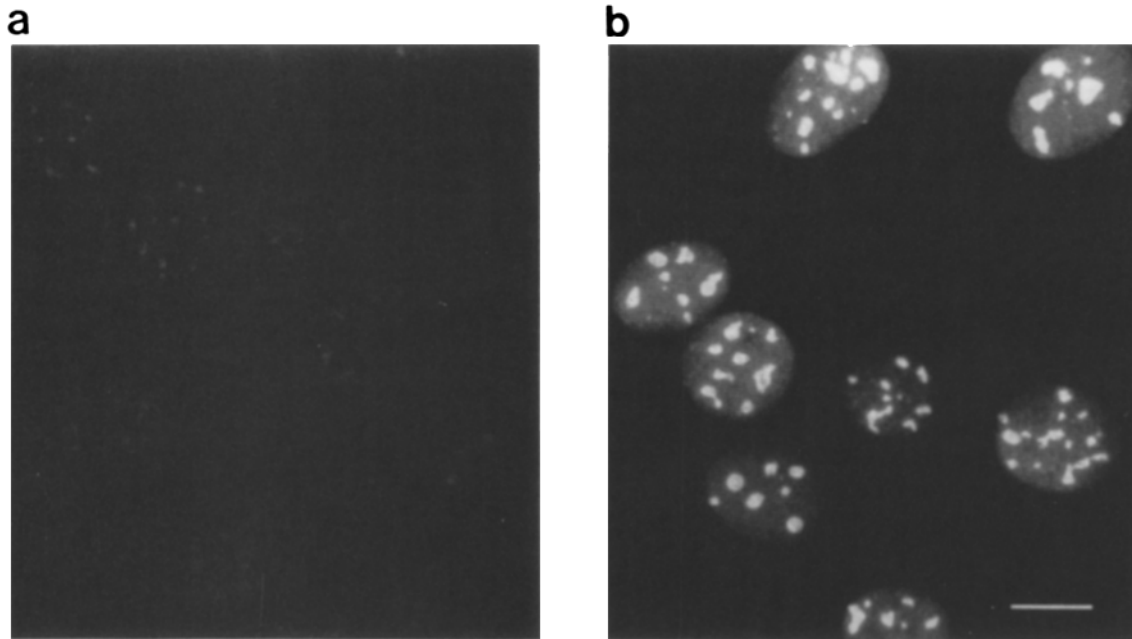


Figure 6. Nuclear localization of the 204 protein: indirect immunofluorescence microscopy of control and IFN-treated AKR 2B cells stained with anti-204 antibodies. Control (a) and IFN-treated AKR 2B cells (b) were fixed, permeabilized, stained with immunoaffinity purified anti-204 antibodies, and the anti-204 antibodies were visualized with fluoresceine-labeled secondary antibodies. For further details see Materials and Methods. Bar, 10 μm .

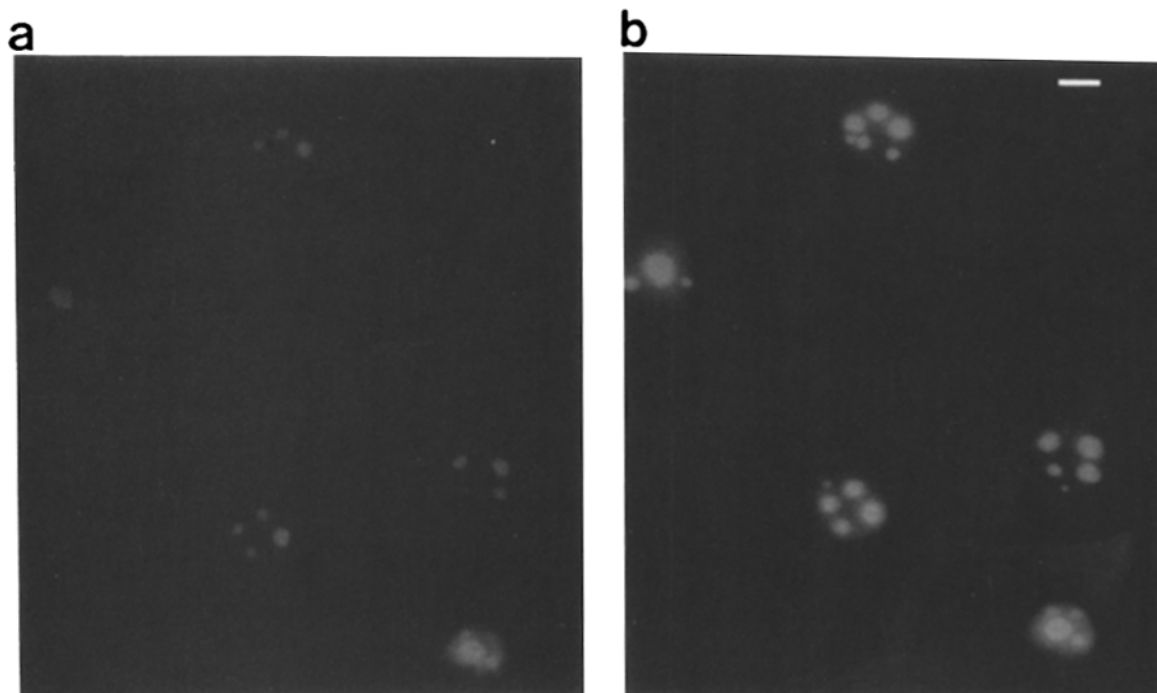


Figure 7. Co-localization of the 204 protein with the nucleolar B23 protein: indirect immunofluorescence microscopy of IFN-treated AKR 2B cells stained with anti-204 antibodies and anti-B23 antibodies. IFN-treated AKR 2B cells were fixed, permeabilized, and stained with immunoaffinity-purified anti-204 antibodies (a) and monoclonal anti-B23 antibodies (b). The anti-204 antibodies were visualized with fluoresceine-labeled secondary antibodies and the anti-B23 antibodies were visualized with rhodamine-labeled secondary antibodies. For further details see Materials and Methods. Bar, 10 μm .

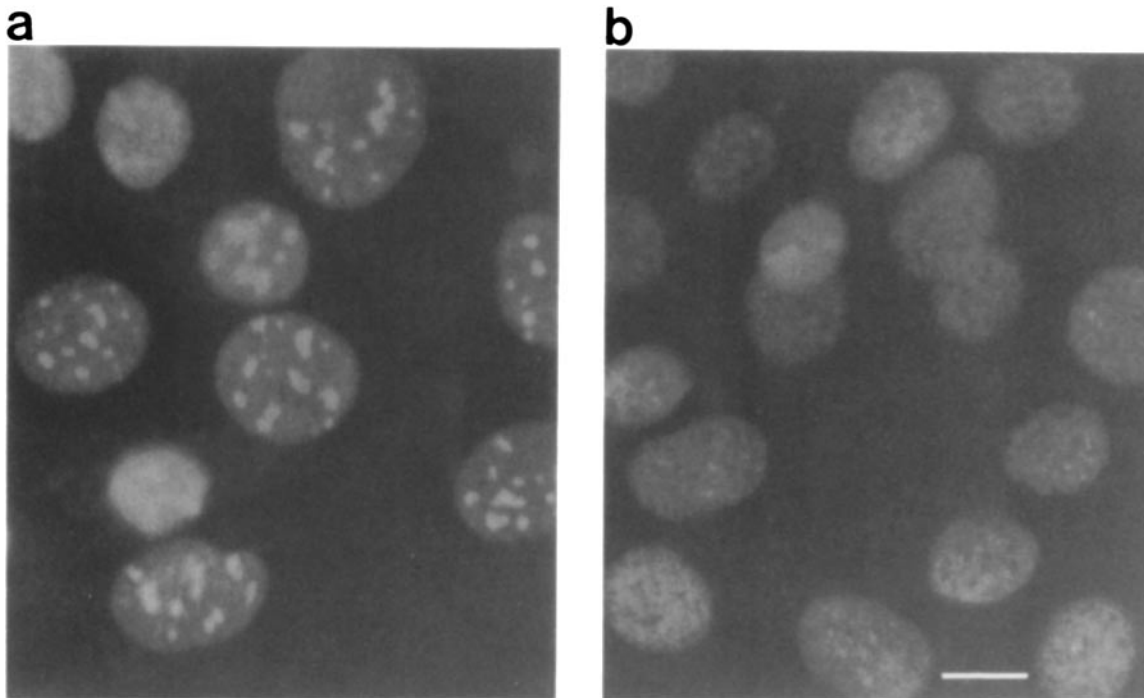


Figure 8. Indirect immunofluorescence microscopy of IFN-treated AKR 2B cells stained with anti-204 antibodies: effect of actinomycin D treatment on the distribution of the 204 protein. IFN-treated AKR 2B cells without (a) or after treatment with actinomycin D (1 $\mu\text{g}/\text{ml}$; Sigma Chemical Co.) for 1 h (b) were fixed, permeabilized, and stained with immunoaffinity purified anti-204 antibodies and the antibodies were visualized with fluoresceine-labeled secondary antibodies. For further details see Materials and Methods. Bar, 10 μm .

amounts of 204 protein solubilized as well as the amounts retained in the residual nuclear fractions after the incubations were determined by SDS-PAGE and immunoblotting. Most of the 204 protein was released by 0.2 M NaCl (not shown). Treatment with DNase 1, (resulting in the degradation of the bulk of the chromosomal DNA into fragments of 2 kb or smaller; not shown) or treatment with RNase A (at concentrations up to 1.5 mg/ml; not shown), however, did not result in the release of detectable amounts of 204 protein.

Discussion

We have generated a polyclonal rabbit antiserum against the NH_2 -terminal segment of the 204 protein. In western blotting and immunoprecipitation assays the antiserum specifically recognized a 72-kD protein in total cell extracts from several murine cell lines. This protein was identified as the 204 protein by (a) its co-migration in SDS-PAGE with 204 protein translated in a reticulocyte lysate driven by an *in vitro* transcript of 204 cDNA and (b) the co-migration of the products of partial proteolytic digestion of the 72-kD protein with those obtained from the 204 protein synthesized *in vitro*. This co-migration of the 204 protein formed *in vivo* with that synthesized *in vitro* makes it likely that no large groups were attached to the protein in the course of posttranslational processing. However, an experiment involving immunoprecipitation from an extract of IFN-treated ^{32}P -labeled AKR 2B cells revealed that the 204 protein is phosphorylated. The site(s) of phosphorylation and the phosphorylating enzyme(s) remain to be identified. The 204 protein amino acid sequence

contains potential sites for phosphorylation by cAMP-dependent kinase, protein kinase C, protein-tyrosine kinase, as well as casein-kinase II (Kemp and Pearson, 1990). Several nucleolar phosphoproteins are known to have phosphorylation sites for casein-kinase II (Olson, 1990).

The basal level of 204 protein in growing AKR 2B cells was low and exposure to IFN resulted in an increase in this level: this was detectable after a 6-h exposure, ~ 50 -fold after 24 h and ~ 75 -fold after 48 h.

We have reported earlier that (a) treatment with IFN increases the level of several mRNAs specified by genes from the 200 cluster (e.g., 202 mRNA, 203 mRNA) (Engel et al., 1988) and (b) as demonstrated in the case of the 202 gene, this increase in mRNA level is in consequence of an increased rate of transcription (Engel et al., 1985). Experiments involving northern blotting (not shown) established that the treatment of AKR 2B cells with IFN strongly enhanced the level of 204 mRNA, with similar kinetics as that of 202 mRNA. This finding is in line with the remarkable sequence similarity of the 5' flanking regions of the 202 and 204 genes (97% in an ~ 0.7 -kb-long segment) (Choubey et al., 1989). These results make it likely that the treatment with IFN increased the level of the 204 protein primarily by increasing the level of 204 mRNA.

A comparison of extracts from several IFN-treated murine cell lines revealed similarly high inducibilities of the 204 protein by IFN in all, except one line – BLKS. In control cultures from this line, the 204 protein was barely detectable and treatment with IFN increased the level only to that in control AKR 2B cells. The molecular basis of this impair-

ment remains to be established. It is relevant, however, that in the BLKS cell line (in which the IFN inducibility of some mRNAs is unimpaired) the 202 protein (Choubey and Lengyel, manuscript in preparation) and 202 mRNA (which are similar in sequence to the 204 protein and the 204 mRNA) remain undetectable even after treating the cells with IFN (Toniato, Choubey, and Lengyel; unpublished data). Moreover, the defect in 202 gene expression is manifested also in the inbred strain of mice (C57 BL/6) from which the BLKS cell line originated. The mechanism of this impairment of 202 gene expression has been explored elsewhere (Gariglio et al., 1992).

Two approaches were followed for determining the subcellular location of the 204 protein: (a) fractionation of AKR 2B cell lysates, coupled with immunoblotting, and (b) indirect immunofluorescence microscopy. Both approaches indicated that the large majority of the protein was distributed between the nucleoli and the nucleoplasm. Co-localization of the 204 protein with B23, a known nucleolar protein (Lischwe et al., 1979), supported this nucleolar assignment.

As revealed by SDS-PAGE analysis (not shown), the 204 protein is a minor component of nucleolar proteins even in cells treated with IFN. It was not detected by Coomassie blue staining, perhaps in consequence of masking by major nucleolar proteins.

The NH₂-terminal region of the 204 protein has several short sequences rich in basic amino acid residues: e.g., -Lys-Lys-Ser-Lys-Ala-Ala-Lys- and -Lys-Lys-Glu-Arg-. Similar sequences have been shown to serve as nuclear targeting signals in other proteins. (Nigg et al., 1991; Silver, 1991). Seven amino acids and 39 amino acids in the COOH-terminal direction from the -Lys-Lys-Glu-Arg- sequence the 204 protein contains putative casein kinase II phosphorylation sites (-Thr-Ser-Leu-Glu- and -Thr-Gln-Glu-Glu-, respectively). Such sites flanking nuclear targeting signals were proposed to increase the rate of transport of proteins into the nucleus (Rihs et al., 1991).

The incubation of nuclei isolated from IFN-treated cells with 0.2 M NaCl released the 204 protein indicating that it is not a component of the nuclear scaffold. DNase I treatment (resulting in the cleavage of DNA into segments shorter than 2 kb), however, did not release detectable quantities of the protein suggesting that it is not associated with DNA. Similarly, treatment with RNase A (at a concentration as high as 1.5 mg protein/ml) did not release 204 protein either, making it probable that this protein resides in a nuclease-resistant compartment. RNase A treatment was shown to release some other nucleolar proteins (e.g., significant amounts of nucleoline (Olson and Thompson, 1983) and small amounts of fibrillarlin (Ochs et al., 1985). The 204 protein resembles a few of the major nucleolar proteins in lacking acidic or glycine-rich segments and known RNA-recognition motifs (Olson, 1990).

Incubation of IFN-treated cells with actinomycin D changed the immunofluorescence pattern of the 204 protein from a nucleolar and punctate version into a more dispersed nucleoplasmic version. This reveals that the protein is associated with components of the nucleoli which become disintegrated after actinomycin D treatment. The treatment was found earlier to result in the translocation of the HeLa B23 protein from the nucleoli to the nucleoplasm (Yung et al., 1985, 1990) without affecting the nucleolar location of some

other proteins, e.g., C23 and fibrillarlin (Chan et al., 1985). Actinomycin D is known to inhibit RNA synthesis, the mechanism by which it induces the translocation of nucleolar proteins into the nucleoplasm however remains to be explored.

Though the large majority of the 204 protein was localized in nuclei, it remains to be examined whether the protein shuttles between the nucleus and the cytoplasm. Some major nucleolar proteins (e.g., nucleolin and B23) were shown to shuttle (Borer et al., 1989; see also Meier and Blobel, 1990).

The gene specifying the 204 protein is part of the 200 cluster of at least six, IFN-activatable genes encoding proteins with very pronounced sequence similarity (Opdenakker et al., 1989; Choubey et al., 1989). The subcellular locations of the proteins other than 204 specified by the 200 cluster remain to be established. It may be relevant to note that the four similar proteins ([2'-5'] oligoadenylate synthetases) specified by another IFN-activatable gene family, were shown to reside in different subcellular compartments (Chebath et al., 1987).

The 204 protein is the first among the IFN-induced proteins to be localized in the nucleolus. The various activities of IFNs (e.g., antiviral, immunomodulatory, and cell growth regulatory) are mediated by the various proteins the IFNs induce. The biological roles of the 204 protein in control and in IFN-treated cells remain to be established. Its nucleolar location makes it possible that it is affecting steps in the biogenesis of ribosomes (Scheer and Benavente, 1990; Reeder, 1990) but antiviral and/or cell growth regulatory activities are also conceivable.

We have transfected into murine cells a 204 cDNA expression construct driven by a strong enhancer. The constitutive overexpression of the 204 protein appeared to impair cell proliferation.

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