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Double-Stranded RNA-Dependent Protein Kinase and 2-5A System Are Both Activated in Interferon-Treated, Encephalomyocarditis Virus-Infected HeLa Cells

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Activation of the interferon-inducible, double-stranded RNA-dependent protein kinase was monitored in monolayer cultures of control and interferon-treated HeLa cells infected with encephalomyocarditis virus. The extent of phosphorylation in the intact cell of the α-subunit of eucaryotic protein synthesis initiation factor eIF2 by the kinase was determined for the first time in this type of system, using a two-dimensional immunoblot technique. Virus protein synthesis and the kinetics of activation of the ppp(A2'p)$_n$A ($n \geq 2$) system were analyzed in parallel. Enhanced phosphorylation of eIF2-α was obvious at 9 h and increased by 12 h postinfection. ppp(A2'p)$_n$A and ppp(A2'p)$_n$A-mediated rRNA cleavage were observed from 6 h. No viral protein synthesis was detected in cells in which a general inhibition of protein synthesis developed with time. It can be concluded that both the kinase and ppp(A2'p)$_n$A system are active in interferon-treated, encephalomyocarditis virus-infected HeLa cells.

There is no single mechanism by which interferon (IFN) treatment of cells inhibits the replication of all viruses. However, protein synthesis is unusually sensitive to inhibition by double-stranded RNA (dsRNA) in extracts from interferon-treated cells (8). Two mechanisms have been identified (reviewed in reference 12). In the first, dsRNA activates the synthetase that synthesizes a series of 2'-5'-linked adenylate oligomers [ppp(A2'p)$_n$A; $n \geq 2$], collectively called 2-5A. This activates a latent cellular endoRNase (2-5A-dependent RNase, RNase L or F) which cleaves mRNA and rRNA. A 2',5'-phosphodiesterase is responsible for degrading 2-5A. Constitutive levels of all of these enzymes are present in variable amounts in a variety of cells and tissues. The level of the 2-5A synthetase increases substantially (10- to 10,000-fold) in response to IFN. The second mechanism involves an IFN-induced dsRNA-dependent protein kinase capable of phosphorylating the α-subunit of the eucaryotic protein synthesis initiation factor eIF2, with a consequent inhibition of protein synthesis (11, 19, 26). It also phosphorylates histones and an endogenous 69-kilodalton (69K) to 72K (human) or 67 to 69K (mouse) polypeptide. These latter may be autophosphorylations, because purification has failed to separate the kinase activity from the 69 to 72K or 67 to 69K polypeptide substrates (7, 9, 21).

Both the protein kinase and 2-5A systems have been widely assayed in cell-free extracts. However, much less has been done to establish whether they are indeed active in the intact IFN-treated, virus-infected cell. Preexisting 2-5A has been extracted from cells and measured by radiobinding and functional assays; evidence for the prior activation of the 2-5A-dependent RNase has been obtained by monitoring for a characteristic pattern of rRNA cleavage (10, 15, 18, 23, 25). In this study we have employed a two-dimensional immunoblot technique (3) to measure the extent of phosphorylation of eIF2-α after IFN treatment and encephalomyocarditis (EMC) virus infection in HeLa cells. Increased phosphorylation can be taken as evidence for activation of the dsRNA-dependent protein kinase in the intact cell (see below). We have also assayed in parallel activation of the 2-5A system and the inhibition of host and viral protein synthesis.

HeLa cells (S3; Flow Laboratories, Inc.) were grown in monolayer culture in Dulbecco modified Eagle medium with 10% heat-inactivated newborn calf serum. Subconfluent plates (30 or 90 mm) of cells treated for 20 h with the indicated amount of human lymphoblastoid (Namalva) cell IFN ($\geq 10^8$ reference units [r.u.] per mg of protein; Wellcome Research Laboratories) (1) were infected with EMC virus (1.4×10^9 PFU/ml) at 20 PFU/cell. Cells (30-mm dishes) were labeled for 30 min with 50 μCi of [^{35}S]methionine (Amersham International; 1,200 Ci/mmol) per ml and processed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (18). 2-5A was extracted with 10% trichloroacetic acid (18) and assayed by a radiobinding assay (10) with ppp(A2'p)$_3$A[^{32}P]pCp (3,000 Ci/mmol; Amersham). Concentrations of 2-5A are given in AMP equivalents. To monitor rRNA cleavage, total RNA was extracted from intact cells with phenol-chloroform (23) and analyzed by gel electrophoresis (18). To measure phosphorylation of eIF-2α, cell lysates were electrophoresed in two-dimensional gels, transferred to nitrocellulose paper, and probed with purified antibodies (3). That any such phosphorylation reflected the situation in the intact cell and not phosphorylation during the extraction procedure was confirmed by control experiments. No phosphorylation was observed when dsRNA (polyinosinic acid-polycytidylic acid; final concentration, 1 μg/ml) was added to mock-infected, IFN-treated cells or when untreated, EMC virus-infected cells were mixed with mock-infected, IFN-treated cells at the time of cell lysis.

Protein synthesis and activation of the protein kinase and 2-5A system were monitored in parallel with different samples of the same cells in the same experiment. In the absence of IFN treatment, protein synthesis was reduced to 15% of that of uninfected cells at 6 h postinfection (p.i.) (Fig. 1A), at

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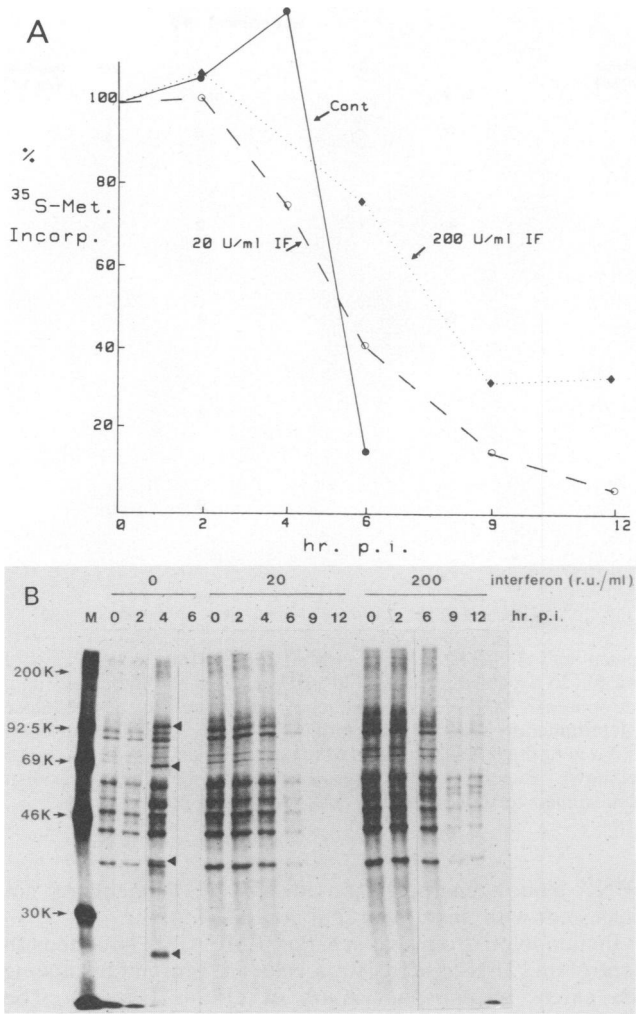

FIG. 1. Time course of protein synthesis in control and IFN-treated, EMC virus-infected HeLa cells. Cells treated with 0 (●), 20 (○), or 200 (◆) r.u. of IFN per ml for 20 h before infection (20 PFU/cell), were pulse-labeled for 30 min with [^{35}S]methionine, and cytoplasmic extracts were prepared. (A) Incorporation of [^{35}S]methionine into protein was determined by trichloroacetic acid precipitation. Values are given as precentages of the values (7,125 cpm [control], 13,316 cpm [20 r.u./ml], 16,313 cpm [200 r.u./ml]) for uninfected cells with corresponding IFN treatment. (B) Labeled cell extracts were analyzed by electrophoresis in a sodium dodecyl sulfate–10% polyacrylamide gel. A fluorograph (Amplify; Amersham International) of the dried gel is shown. Extracts from ca. 2.5 × 10^4 cells were loaded in each lane. The molecular weights of marker polypeptides (lane M) are indicated by arrows to the left of the gel. The major EMC virus polypeptides also are indicated (◀).

which time cell lysis was first observed. Virtually all cells had lysed by 12 h. EMC virus protein synthesis was well established at 4 h p.i. In EMC virus-infected cells pretreated with 20 r.u. of IFN per ml, some cell lysis was observed at 12 h p.i. No cell lysis was observed up to 12 h in cells pretreated with 200 r.u. of IFN per ml. No EMC virus-specific polypeptides were detected in these IFN-treated cells (Fig. 1B).

In control HeLa cells, which have a high basal level of the enzyme 2-5A synthetase without IFN treatment (23), 2-5A and rRNA cleavage were first detected at 6 h p.i. (Fig. 2). Limited activation of the 2-5A system in control cells is consistent with the results observed in a previous study in which HeLa cells were grown in suspension rather than as monolayers. Its significance and the inhibition of the 2-5A-dependent RNase by EMC virus infection in the absence of

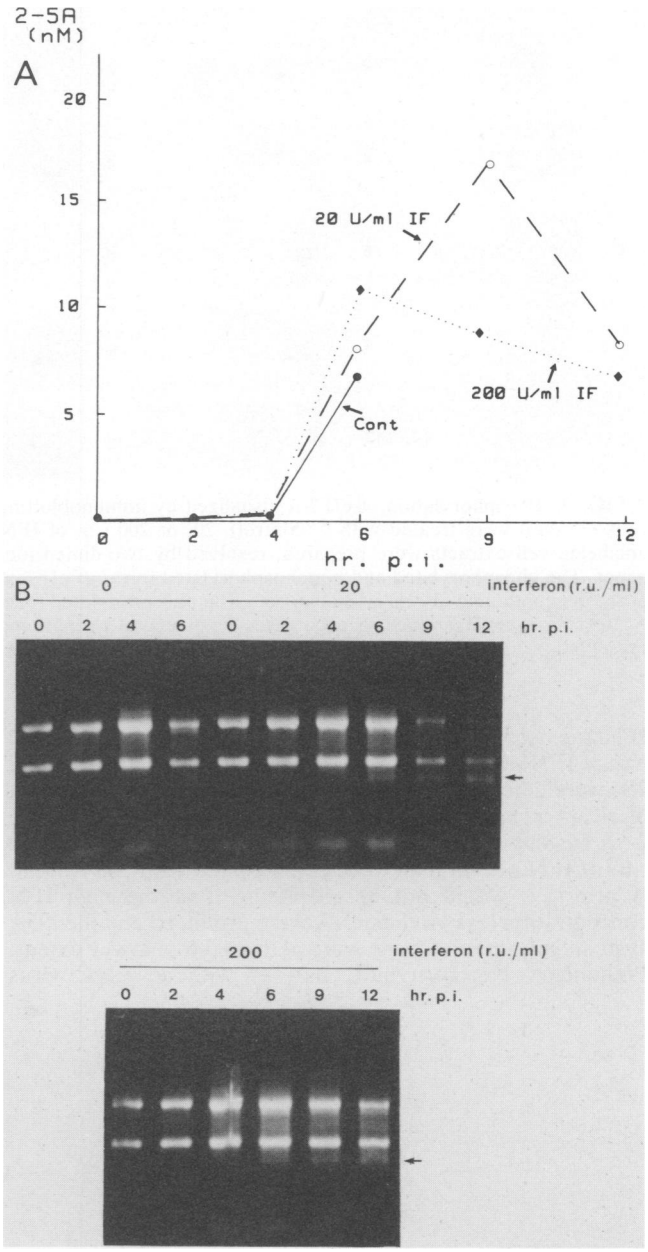

FIG. 2. Time course of 2-5A accumulation (A) and rRNA cleavage (B) in control and IFN-treated, EMC virus-infected HeLa cells. Plates of cells (90 mm) were treated with 0 (●), 20 (○), or 200 (◆) r.u. of IFN per ml for 20 h before infection (20 PFU/cell). (A) Trichloroacetic acid extracts were prepared at indicated time points and assayed for 2-5A by the radiobinding assay (10). The intracellular 2-5A (ordinate) concentrations were calculated from those in the extracts by assuming 100% recovery; the actual recovery of added 2-5A in this type of experiment was ≥50%. (B) Total cellular RNA was extracted at the indicated time points and electrophoresed in a 1.6% neutral agarose gel; 20% of the recovered RNA (ca. 20 μg) from each extraction was loaded in each lane. A photograph of the ethidium bromide-stained gel is shown. A 2-5A-specific rRNA cleavage product below 18S rRNA is indicated by an arrow.

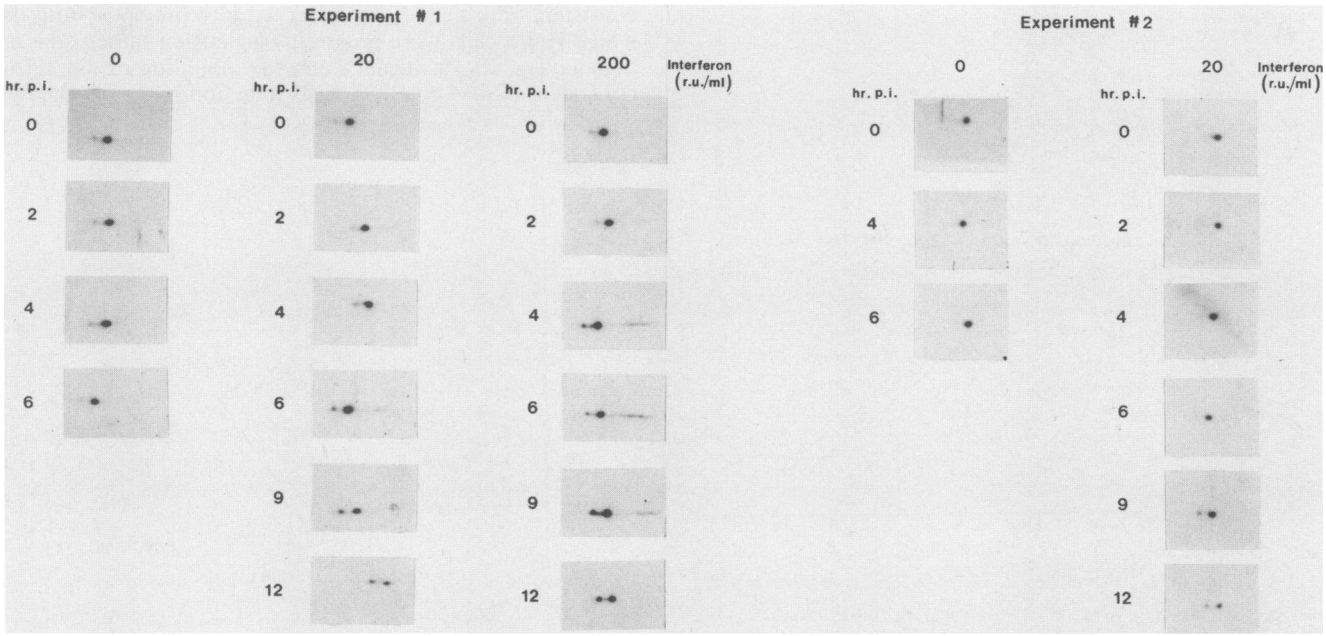

FIG. 3. Phosphorylation of eIF2-α visualized by immunoblotting in control and IFN-treated, EMC virus-infected HeLa cells. Plates of cells (30 mm) were treated with 0 (control), 20, or 200 r.u. of IFN per ml for 20 h before infection (20 PFU/cell). At the indicated time, unlabeled cell extracts were prepared, resolved by two-dimensional electrophoresis, and electrophoretically transferred to nitrocellulose paper. The phosphorylated and nonphosphorylated forms of eIF2-α were determined by reaction with antibodies to eIF2-α. Only the sector of the gel containing eIF2-α was analyzed. The phosphorylated form of eIF2-α is to the left side (acidic) of the gel. Cell extracts from ca. 2.5 × 10^5 cells were examined in each case. Two separate experiments are shown. Experiment no. 1 was from the same infection as that presented in Fig. 1 and 2. For the infection shown in experiment no. 2, data similar to those in Fig. 1 and 2 were obtained.

IFN has been discussed (23). In cells treated with 20 or 200 r.u. of IFN per ml, 2-5A (Fig. 2A) and rRNA cleavage (Fig. 2B) were detected from 6 h p.i. The level of 2-5A and extent of rRNA cleavage suggests there is a slightly more active 2-5A system in EMC virus-infected cells pretreated with 20 r.u. of IFN per ml than in cells pretreated with 200 r.u./ml. A priori, it would not be surprising if at the high IFN concentration less viral dsRNA were produced and the 2-5A system and protein kinase were activated to a lower extent. Preliminary Northern blot analyses with an EMC virus cDNA clone (generously provided by A. Palmenberg) are consistent with this notion (but contrast Fig. 4).

Immunoblots from two separate EMC virus infections in control and IFN-treated HeLa cells are presented showing the extent of phosphorylation of eIF2-α (Fig. 3). The phosphorylated form of eIF2-α is to the left (acidic side). Relative levels of phosphorylation of eIF2-α were quantitated by scanning the original X-ray film (Fig. 4). The reason for the difference in background phosphorylation before infection in the two experiments is unclear, because in both

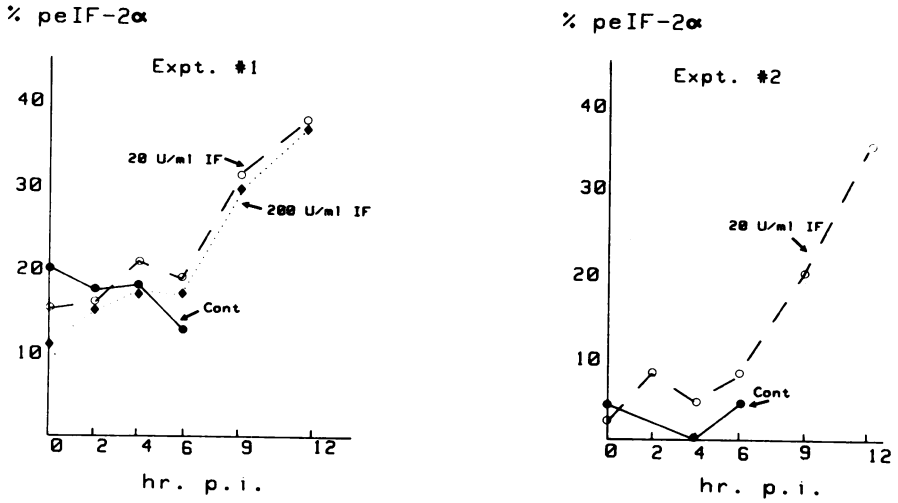

FIG. 4. Quantitation of the level of phosphorylation of eIF2-α in control and IFN-treated, EMC virus-infected HeLa cells. The immunoblots shown in Fig. 3 were quantitated by scanning the original X-ray film with a Joyce Loebel Chromoscan 3 densitometer.

experiments the cells were subconfluent and in exponential growth. In the absence of IFN treatment there was no increased phosphorylation of eIF2-α during EMC virus infection (Fig. 3 and 4). In IFN-treated, EMC virus-infected cells, increased phosphorylation of eIF2-α was first detected at 9 h p.i. and increased by 12 h. Quantitation of the immunoblots indicates that the level of eIF2-α phosphorylation increases from a basal level of about 10% in uninfected cells to 30 to 40% at 12 h p.i. in IFN-treated cells (Fig. 4). A 30% phosphorylation of eIF2-α is correlated with the complete inhibition of protein synthesis in rabbit reticulocyte lysates. This is thought to be because phosphorylated eIF2-α irreversibly binds to and inactivates a second rate-limiting factor called eIF2B, RF, or GEF (22). It is reasonable to assume that the same will hold true for HeLa cells, although this remains to be firmly established. The use of the immunoblotting technique with additional affinity-purified antibodies (3) revealed no significant alterations in eIF2-β, eIF4A, and eIF4B (data not shown).

We are aware of only three reports concerning activation of the IFN- and dsRNA-dependent protein kinase in intact cells. Phosphorylations of the 67 to 69K polypeptide and eIF2-α, respectively, were observed in IFN-treated, reovirus infected L_{929} (6) and HeLa (14) cells. In contrast, in IFN-treated L_{929} cells no such phosphorylation was detected with either mengovirus or vesicular stomatitis virus (5). It was concluded that it is unlikely that the kinase is involved in the antiviral action of IFN against these latter viruses. Here we present evidence for the activation in the intact cell of both the kinase and the 2-5A system in IFN-treated, EMC virus-infected HeLa cells (Fig. 2, 3, and 4). The difference between our results and those for the kinase with the very closely related mengovirus presumably reflects differences either in the cells (HeLa versus L_{929}) or in the culture conditions or experimental procedures employed. Activation of the 2-5A system and protein kinase was first detected by the methods employed in this study at 6 h p.i. or later (Fig. 2, 3, and 4), whereas in the absence of IFN treatment virus protein synthesis was well established by 4 h (Fig. 2). On the face of it, therefore, their activation occurred too late for them to be involved in the inhibition of early stages in virus replication. On the other hand, earlier low-level or localized (2, 13) activation cannot be excluded. Even if an earlier activation of these systems were detected, the significance of the activation in mediating the inhibition of virus growth would remain to be established. Similarly, no rigorous conclusions can be drawn concerning the importance of these systems in the inhibition of protein synthesis late in infection (Fig. 1). It would be extraordinary, however, if they were not in some way involved.

Attempts by a number of groups to correlate sensitivity to interferon for different viruses with the presence or absence of the kinase or one or other of the enzymes of the 2-5A system, although highly suggestive in some cases, have been generally inconclusive. In no case has it been possible to establish rigorously the absence of enzyme activity in the intact cell. Results, for example, with the NIH/3T3 clone I cells, originally thought to be deficient in the 2-5A-dependent RNase, have been very different in different laboratories (4, 16). Similarly, although inactivation of the kinase by vaccinia virus correlates with the ability of this virus to rescue vesicular stomatitis virus replication from inhibition by IFN (17, 24), vaccinia virus infection also affects the 2-5A system (17, 18) and may affect the expression of the IFN response in other ways. Resolution of the question of the importance of the kinase and 2-5A systems in the antiviral action of IFN against different viruses will, therefore, have to await future analyses with either rigorously characterized mutants in their function or specific inhibitors of their activation.

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ADDENDUM IN PROOF

The dsRNA-dependent protein kinase has recently been shown to be active in IFN-treated, reovirus-infected cells and results in increased phosphorylation of eIF-2α (20).

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