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Mechanism of interferon action: Phosphorylation of protein synthesis initiation factor eIF-2 in interferon-treated human cells by a ribosome-associated kinase processing site specificity similar to hemin-regulated rabbit reticulocyte kinase

(translational control/double-stranded RNA/reovirus/antiviral agents)

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ABSTRACT **The phosphorylation of purified protein synthesis factors catalyzed by protein kinase preparations isolated from interferon-treated human amnion cells was examined. Ribosomal salt-wash fractions prepared from interferon-treated human cells contained a protein kinase that catalyzed the [γ-^{32}P]ATP-mediated phosphorylation of the 38,000-dalton subunit of eukaryotic initiation factor 2 (eIF-2α); this kinase activity was significantly enhanced in interferon-treated as compared to untreated cells. The tryptic [^{32}P]phosphopeptide pattern obtained for eIF-2α phosphorylated by the interferon-mediated human kinase was indistinguishable from the pattern obtained for eIF-2α phosphorylated by the hemin-regulated rabbit reticulocyte kinase when analyzed by thin-layer chromatography with three different solvent systems and by high-voltage electrophoresis. *O*-[^{32}P]Phosphoserine was liberated by partial acid hydrolysis from eIF-2α phosphorylated by either the human or the rabbit kinase. In addition to the phosphorylation of eIF-2α, interferon treatment of human cells enhanced the phosphorylation of two additional ribosome-associated proteins designated P_I and P_f. The major phosphoester linkage observed for the human, as well as murine, phosphoprotein P_I was *O*-phosphoserine. The interferon-mediated phosphorylation of both eIF-2α and protein P_I was dependent upon the presence of RNA with double-stranded character; P_f phosphorylation was not affected by double-stranded RNA. These results suggest that the interferon-mediated ribosome-associated human protein kinase catalyzes the phosphorylation of eIF-2α in a site-specific manner that is apparently identical with the reaction catalyzed by the hemin-regulated rabbit reticulocyte kinase; hence, the phosphorylation of eIF-2 may play a role in regulating the initiation of translation in interferon-treated cells.**

Post-translational modifications such as protein phosphorylation provide an important mechanism by which the functional activity of proteins can be controlled and, hence, biological processes regulated (1, 2). For example, in rabbit reticulocytes during hemin deficiency, protein synthesis is inhibited by a cyclic AMP-independent protein kinase that catalyzes the phosphorylation of the small subunit of the protein synthesis initiation factor eIF-2 (3, 4). Protein synthesis is also inhibited in reticulocyte lysates in the presence of double-stranded (ds) RNA (5), which appears to activate a protein kinase also capable of catalyzing the phosphorylation of eIF-2 (6, 7). The phosphorylation of eIF-2 significantly reduces its ability to function efficiently at the level of initiation of protein synthesis (7–11).

In many animal virus–host cell systems, the primary level of virus genome expression inhibited as a result of interferon treatment is the translation of viral mRNA into protein (12, 13). The inhibition of protein synthesis observed in cell-free extracts prepared from interferon-treated cells is due, in several systems, to the presence of a ribosome-associated inhibitor (14–17). Interferon treatment also mediates an enhanced protein phosphorylation (18–21). The phosphorylation of certain proteins in the ribosomal salt-wash fraction prepared from interferon-treated cells correlates with the appearance of the ribosome-associated inhibitor of translation (21). The inhibition of translation in interferon-treated cell-free systems occurs both at the level of initiation and of elongation of polypeptide chain biosynthesis. The inhibition at the level of initiation, characterized by a reduction in the association of virus mRNA to ribosomes (22) and a reduction in the formation of virus-specific methionyl-X initiation peptides (23), is accompanied by a reduction in protein synthesis eIF-2 activity (24, 25). The inhibition at the apparent level of elongation appears to be due, in part, to either the nucleolytic degradation of the viral mRNA (26, 27) or to a deficiency of certain minor tRNA species (28, 29) or to both.

The studies reported in this paper characterize in detail the nature of the phosphorylation of eIF-2 by a ribosome-associated protein kinase from interferon-treated human amnion U cells.

EXPERIMENTAL PROCEDURE

Materials. [γ-^{32}P]ATP (specific activity, 30.7 Ci/mmol) was from New England Nuclear; N^{α}-tosyllysine chloromethyl ketone (TPCK)-treated trypsin was from Worthington; cellulose thin-layer chromatography roll (0.1-mm thick layer without fluorescent indicator) was from EM Laboratories (Elmsford, NY); human leukocyte interferon was generously provided by T. C. Merigan (Stanford University Medical Center); hemin-regulated rabbit reticulocyte protein kinase, purified through DEAE-Sephadex, and homogeneous rabbit reticulocyte initiation factor eIF-2 were generously provided by I. M. London and R. S. Ranu (Massachusetts Institute of Technology, Cambridge, MA); and purified rat liver elongation factors EF-1 and EF-2 were generously provided by K. Moldave, (University of California, Irvine, CA). All chemicals were reagent grade.

Ribosomal Salt-Wash Fractions. These fractions were prepared from untreated and interferon-treated (100 units/ml) human amnion U cells as described (30).

Reovirus RNAs. Reovirus genome dsRNA was isolated from purified reovirions (31). Reovirus single-stranded (ss) RNA (mRNA) was prepared as the *in vitro* transcription product (32) and was purified as described (15).

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Abbreviations: dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; eIF, eukaryotic initiation factor; eIF-2α, the small (38,000-dalton) subunit of eIF-2; EF, elongation factor; $NaDodSO_4$, sodium dodecyl sulfate.

***In Vitro* Phosphorylation Assay.** *In vitro* [γ-^{32}P]ATP-mediated phosphorylation assays were performed as described (21) except that the reaction mixture was modified to contain, as indicated: 0.8 M ribosomal salt-wash fraction possessing the human interferon-mediated kinase; hemin-regulated rabbit reticulocyte kinase; purified protein synthesis factors eIF-2, EF-1, or EF-2; and, reovirus ds or ss RNA. The ^{32}P-labeled products were analyzed by sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide slab-gel electrophoresis, and autoradiography was performed with Du Pont Cronex 20 × 25-cm film (21).

Tryptic Peptide Analysis. Phosphoproteins eIF-2α and P_1, labeled with [γ-^{32}P]ATP, were purified by $NaDodSO_4$/polyacrylamide gel electrophoresis. Preparative gels were rinsed very briefly in cold H_2O without fixing or staining, wrapped in Saran Wrap, and exposed to film at 4°C for about 5 hr. After autoradiography, the protein bands corresponding to the α subunit of eIF-2 and to P_1 were cut from the gel with a razor blade with the film as a template. The gel pieces containing eIF-2α and P_1 were crushed with a Teflon rod and the radioactive proteins were eluted with 50 mM NH_4HCO_3 by agitating. The efficiency of elution and the purity of the eIF-2α and P_1 preparations were established from analytical gels and autoradiograms prepared from dried gels. The extracted proteins were digested with trypsin as described (34). Tryptic peptides were analyzed by ascending thin-layer chromatography on plastic-supported cellulose sheets (8 × 18 cm). The sheets were developed in glass jars with the following solvents (by vol): A, acetic acid/1-butanol/pyridine/water (3:15:10:12); B, acetic acid/1-butanol/water (1:4:1); and C, acetic acid/pyridine/water (30:50:15). High-voltage thin-layer electrophoresis was performed on plastic-supported cellulose sheets (40 × 20 cm) with an adapter for a Savant, high-voltage power supply and tank system. Electrophoresis was at pH 4.7 (5% butanol/2.5% pyridine/2.5% acetic acid/water, by vol) for 1.7 hr at 2000 V.

Identification of Amino Acids. Purified phosphoproteins labeled with ^{32}P were partially hydrolyzed in HCl (6 M glass-distilled HCl at 110°C for 2.0 hr under reduced pressure). Hydrolyzed samples were analyzed, together with unlabeled marker *O*-phosphoserine and *O*-phosphothreonine, by electrophoresis on Whatman 3 MM paper in 7% formic acid (pH 1.6) for 3.2 hr at 1250 V. The unlabeled marker phospho amino acids were detected with ninhydrin stain, and the ^{32}P-labeled hydrolysis products were detected by autoradiography.

RESULTS

Effect of Interferon Treatment on Phosphorylation of Ribosomal Salt-Wash Proteins and Purified Protein Synthesis Factors. Ribosomal salt-wash fractions prepared from untreated and interferon-treated human amnion U cells were examined for their ability to catalyze [γ-^{32}P]ATP-mediated phosphorylation of endogenous proteins and of purified eIF-2. Phosphorylation of a component designated P_1 present in the 0.8 M ribosomal wash fraction prepared from cells treated with interferon was greatly enhanced as compared to the phosphorylation obtained with the comparable wash fraction prepared from untreated cells (Fig. 1). Human cells also possessed a smaller ribosome-associated protein, designated P_f, that was phosphorylated in extracts prepared from interferon-treated but not untreated cells. The phosphorylation of P_1 was dependent upon the presence of dsRNA; P_f phosphorylation was not affected by dsRNA.

Purified protein synthesis initiation factor eIF-2 was also incubated with the ribosomal salt-wash fractions prepared from interferon-treated and untreated human cells, and the reaction mixtures were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. eIF-2 is composed of three nonidentical subunits (34). As shown in Fig. 1, the smallest subunit (α, 38,000 daltons) of eIF-2 was phosphorylated by the interferon-mediated human kinase.

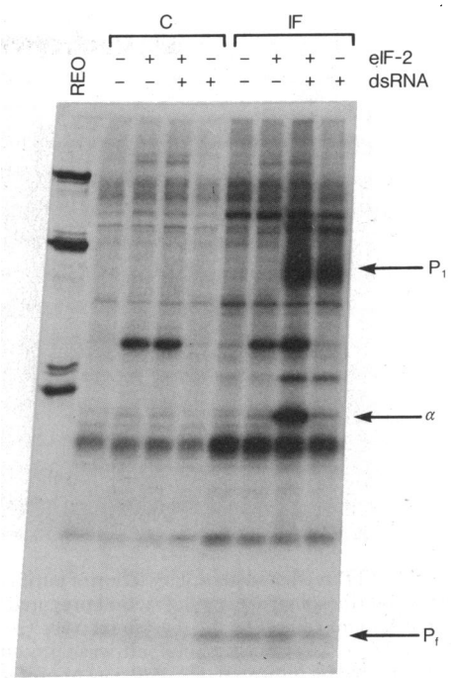

FIG. 1. Autoradiogram showing effect of interferon treatment and dsRNA on phosphorylation of eIF-2α. Phosphorylation was catalyzed by ribosomal salt-wash fraction (2.5 μg of protein) prepared from untreated (C) and interferon-treated (IF) human cells. The incubation mixture (26 μl) contained eIF-2 (0.5 μg of protein) and reovirus genome dsRNA (1 μg/ml) as indicated. Incubation was for 15 min at 30°C. REO, [^{14}C]leucine-labeled reovirion proteins. Arrows, positions of ^{32}P-labeled phosphorylated proteins P_1 and P_f present in the ribosomal salt-wash and of the α subunit of eIF-2.

The phosphorylation of eIF-2α was dependent upon both interferon treatment and dsRNA (Fig. 1). The ribosomal salt-wash preparations from untreated control human cells, in either the presence or absence of dsRNA, catalyzed the phosphorylation of eIF-2α less than 10% as efficiently as salt-wash preparations from interferon-treated cells. The phosphorylation of eIF-2α and of protein P_1 was dependent upon the amount of ribosomal salt-wash protein added to the reaction mixture (Fig. 2). No significant radioactivity was associated with either of the elongation factors, EF-1 or EF-2, when they were examined as substrates for the interferon-mediated kinase (Fig. 2). The purified eIF-2 preparation contained a low level of protein kinase activity that phosphorylates the β subunit of eIF-2 but not the α subunit (ref. 35; see also Fig. 5); the interferon-mediated ribosome-associated human kinase did not significantly phosphorylate eIF-2β (Fig. 2).

The effect of incubation time on the ability of the interferon-mediated human kinase activity to catalyze the phosphorylation of proteins P_1 and P_f and of eIF-2α is shown in Fig. 3. The phosphorylation of P_1 and eIF-2α followed similar kinetics and was maximal by 20 min of incubation under standard reaction conditions; the amount of radioactivity associated with P_1 and eIF-2 did not significantly change after 20 min. By contrast, the accumulation of phosphorylated P_f was somewhat more rapid than P_1 and eIFα and was maximal by 10 min; the amount of radioactivity associated with P_f then decreased upon further incubation.

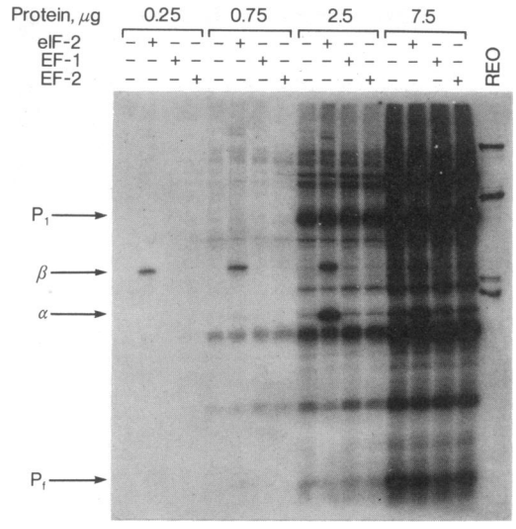

FIG. 2. [γ-^{32}P]ATP-mediated phosphorylation of purified protein synthesis factors by ribosomal salt-wash fraction prepared from interferon-treated human cells. The incubation mixture (26 μl) contained 0.25, 0.75, 2.5, or 7.5 μg of ribosomal salt-wash protein, 1 μg of dsRNA per ml, and where indicated, eIF-2 (0.5 μg of protein), EF-1 (0.5 μg of protein), or EF-2 (1.0 μg of protein). Conditions and symbols as described for Fig. 1.

Fulfillment of dsRNA Requirement by Reovirus ssRNA. The interferon-mediated phosphorylation of protein P_1 and of eIF-2α showed an apparent dependence upon dsRNA (Fig. 1). As shown in Fig. 4, the *in vitro* activation of the interferon-mediated kinase by reovirus genome dsRNA can also be obtained with reovirus ssRNA purified by repeated LiCl precipitation. At a concentration of ssRNA typical of that used in cell-free protein-synthesizing systems, 60 μg/ml (15, 21), the phosphorylation of protein P_1 and of eIF-2α was comparable

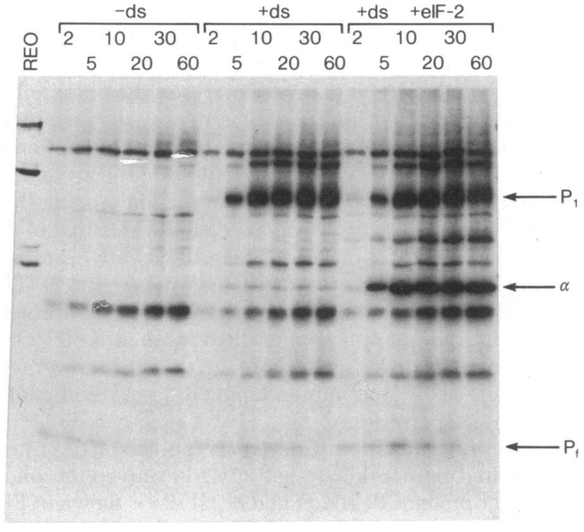

FIG. 3. Kinetics of [γ-^{32}P]ATP-mediated phosphorylation of proteins P_1 and P_f and of eIF-2α catalyzed by ribosomal salt-wash fraction prepared from interferon-treated human cells. The reaction mixture contained 0.8 M ribosomal salt-wash (12.5 μg of protein), reovirus genome dsRNA (1 μg/ml), and eIF-2 (2.5 μg of protein) as indicated. Reaction volume, 125 μl; 15-μl aliquots were processed after incubation at 30°C for 2, 5, 10, 20, 30, or 60 min. Symbols are as described for Fig. 1.

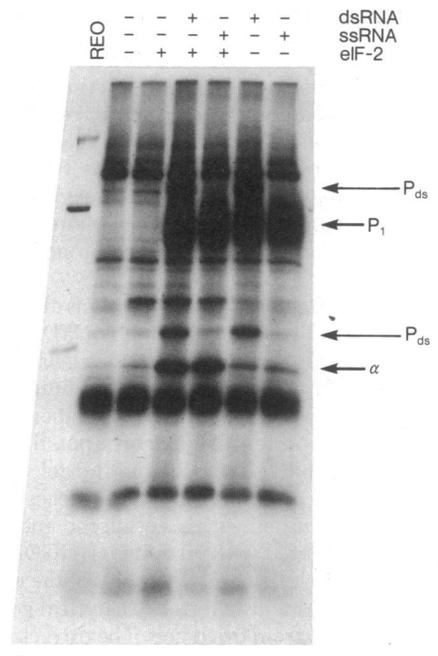

FIG. 4. Effect of reovirus dsRNA and ssRNA on phosphorylation of eIF-2α and of proteins P_1 and P_{ds} present in the 0.8 M ribosomal salt-wash fraction. The incubation mixture contained 2.7 μg of wash fraction protein from interferon-treated human cells and, where indicated, eIF-2 (0.5 μg of protein), reovirus genome dsRNA (1 μg/ml), and reovirus ssRNA (60 μg/ml). Conditions and symbols as for Fig. 1.

to that observed in the presence of reovirus dsRNA. However, the phosphorylation of at least two proteins, designated P_{ds}, was observed in the presence of reovirus dsRNA but not reovirus ssRNA (Fig. 4). The dsRNA-dependent phosphorylation of the proteins designated P_{ds} was not dependent upon interferon treatment; the P_{ds} phosphorylations were observed in untreated human and murine cells as well as in interferon-treated cells (results not shown).

Phosphorylation of eIF-2α by Interferon-Mediated Human Kinase and by Hemin-Regulated Rabbit Reticulocyte Kinase. The interferon-mediated, ribosome-associated, human U cell kinase, which is dependent upon dsRNA for activation, and the hemin-regulated rabbit reticulocyte kinase, which is not dependent on dsRNA, both catalyze the phosphorylation of the α subunit of eIF-2 (Fig. 5).

In order to establish the structural relationship between eIF-2α phosphorylated by the interferon-mediated kinase as compared to the hemin-regulated kinase, tryptic [^{32}P]peptides derived from the α subunit were compared. The products of trypsin digestion of ^{32}P-labeled eIF-2α phosphorylated by either the human U cell or the rabbit reticulocyte kinase, when characterized by thin-layer chromatography on cellulose plates with three different solvent systems, migrated with comparable R_F values (Fig. 6). By high-voltage electrophoresis, the phosphopeptides derived from eIF-2α by phosphorylation with either the interferon-mediated or the hemin-regulated kinase migrated toward the anode with similar mobilities (Fig. 7). Similarity in the modification of eIF-2α by the two kinases was confirmed by chromatographic and electrophoretic analyses of mixtures of the peptides of the two preparations (Figs. 6 and 7). The multiple radioactive components observed in each preparation may represent either partial proteolysis products of eIF-2α or multiple sites of phosphorylation.

To establish the nature of the phosphoester linkage in phos-

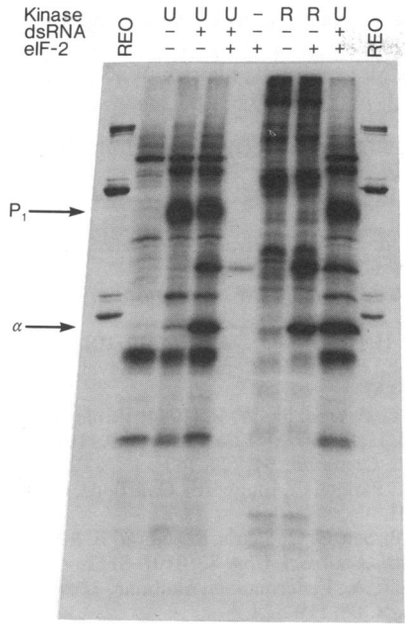

FIG. 5. Phosphorylation of eIF-2α catalyzed by interferon-mediated, ribosome-associated human kinase (U) and the hemin-regulated rabbit reticulocyte kinase (R). The reaction mixture contained eIF-2 (0.5 μg of protein), dsRNA (1 μg/ml), and U (2.5 μg of protein) or R (2.0 μg of protein) kinase preparations as indicated. Incubation was for 20 min at 30°C.

phorylated eIF-2α and protein P_1, we subjected the phosphoproteins to partial acid hydrolysis and analyzed the hydrolysates by high-voltage paper electrophoresis. The ^{32}P-labeled material liberated from eIF-2α phosphorylated by both the interferon-mediated and the hemin-regulated kinases migrated either with unlabeled *O*-phosphoserine marker or with inorganic phosphate (Fig. 8). In addition, the major phosphoester observed after partial acid hydrolysis of both human U and murine L_{929} phosphoprotein P_1 was likewise *O*-phosphoserine, although low levels of *O*-phosphothreonine were also detectable in both P_1 preparations (Fig. 8).

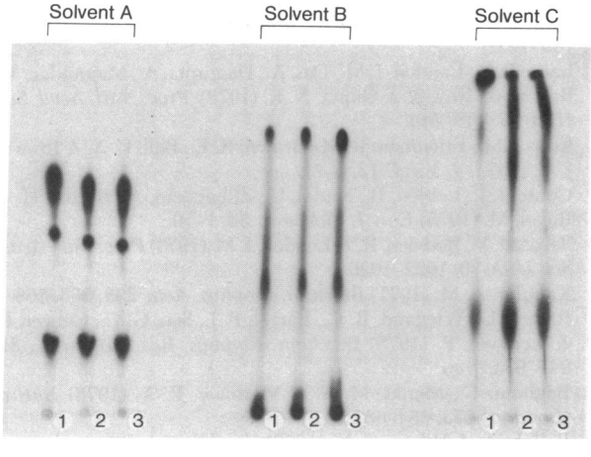

FIG. 6. Chromatographic characterization of the tryptic [^{32}P]-peptides of eIF-2α phosphorylated by either the interferon-mediated human U cell kinase (track 1) or the hemin-regulated rabbit reticulocyte kinase (track 2). The ^{32}P-labeled α-subunit band of eIF-2 was excised from a preparative gel, extracted, digested with trypsin, and analyzed by thin-layer chromatography with three different solvent systems. Track 3, U cell kinase plus reticulocyte kinase.

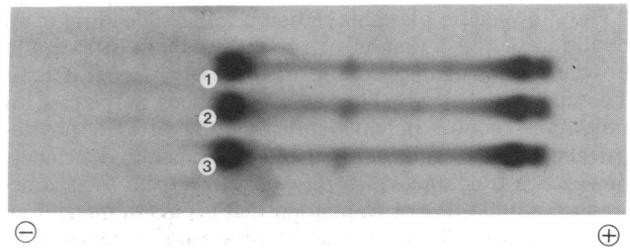

FIG. 7. Electrophoretic characterization at pH 4.7 of the tryptic peptides of eIF-2α phosphorylated by either the interferon-mediated human kinase (track 1) or the hemin-regulated reticulocyte kinase (track 2). Track 3, human kinase plus reticulocyte kinase.

DISCUSSION

The results reported here establish that a ribosome-associated protein kinase present in interferon-treated human cells catalyzes the phosphorylation of the small subunit (α) of protein synthesis initiation factor eIF-2 in a site-specific manner that is structurally indistinguishable from the eIF-2α phosphorylation catalyzed by the hemin-regulated rabbit reticulocyte protein kinase. Interferon-treated intact cells show a reduced ability to support the initiation of viral protein synthesis *in vivo* (22). *In vitro* studies with cell-free systems have likewise revealed that the initiation of translation is affected by interferon treatment (23, 24, 36, 37). In the rabbit reticulocyte system, the phosphorylation of eIF-2α is a necessary condition for the inhibition of protein synthesis at the initiation level (7–11). It appears that in interferon-treated cells the reduction in initiation of translation (22–24, 36, 37) may also be due, in part, to the phosphorylation of eIF-2α. The appearance of the interferon-mediated protein kinase associated with the ribosomal salt-wash fraction correlates with the appearance of ribosome-associated inhibitor of reovirus mRNA translation (21), and the inhibition of translation caused by the interferon-mediated kinase can be reversed, in part, by the addition of eIF-2 (25).

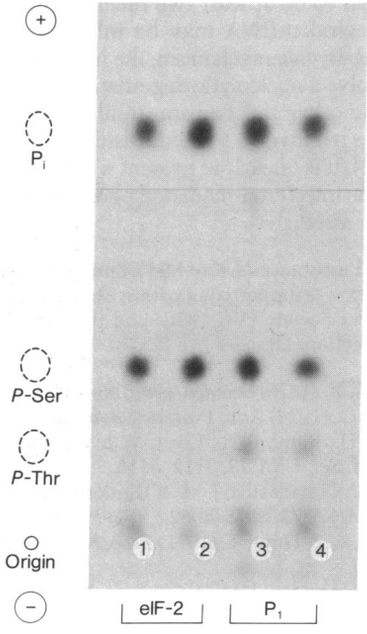

FIG. 8. Electrophoretic identification of phosphoserine in the ^{32}P-labeled α subunit of eIF-2 and in protein P_1. Hydrolysis products of purified eIF-2α phosphorylated with either the human interferon-mediated kinase (track 1) or the hemin-regulated kinase (track 2), and of P_1 phosphorylated in ribosomal salt-wash fractions from interferon-treated human (track 3) and murine (L_{929}) (track 4) cells, were analyzed.

The site-specific phosphorylation of eIF-2α, leading to an inhibition of the initiation of protein synthesis, appears to provide another example of the existence in different systems of common or analogous biochemical reactions, in this case for translational control in two seemingly quite different physiological conditions, interferon treatment and hemin deficiency. However, a fundamental difference does appear to exist between the inhibition of translation that occurs in interferon-treated animal cells maintained in culture and in hemin-deficient rabbit reticulocytes. In interferon-treated cells the inhibition of protein synthesis is characterized by a discrimination between cellular and viral mRNAs (12, 13), whereas in hemin-deficient reticulocytes the synthesis of almost all proteins, including globin, appears to be inhibited (38). The explanation as to how the modification by phosphorylation of eIF-2, a normal component of the host cells' translational machinery, could contribute to an apparent discrimination between viral and cellular mRNA translation inhibition in interferon-treated cells is not clear.

The apparent requirement for dsRNA in the activation of the interferon-mediated kinase could conceivably be fulfilled *in vivo* by dsRNA regions of mRNA resulting from the secondary structure of the ssRNA or, alternatively, by dsRNA *per se* produced during replication of RNA virus genomes or by symmetrical transcription of dsDNA. For example, reovirus ssRNA contains significant ds regions, as indicated by the binding of ethidium bromide (39); reovirus ssRNA also activates the phorphorylation of eIF-2α and protein P_1 in interferon-treated extracts (Fig. 4). Although the possibility of trace dsRNA genome contamination of the ssRNA message cannot be entirely excluded, activation of both untreated and treated washes with genome dsRNA resulted in the enhanced phosphorylation of proteins not observed with message ssRNA (Fig. 4). The differential effects of reovirus dsRNA and ssRNA on protein phosphorylation suggest that either different kinase or phosphatase activities or both may be affected by the viral RNAs.

There appear to be at least two mechanisms by which the translation of viral mRNA may be inhibited in interferon-treated systems. By one mechanism, the inhibition of translation appears to involve a nucleolytic degradation of the message (27, 40, 41); by the other mechanism, inhibition of viral mRNA translation does not involve a detectable degradation of the viral mRNA (22, 42). It is unclear at present with which of the above mechanisms the interferon-mediated phosphorylation of eIF-2α is most closely associated.

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