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The Phosphorylation State of Eucaryotic Initiation Factor 2 Alters Translational Efficiency of Specific mRNAs

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Phosphorylation of the alpha subunit of the eucaryotic translation initiation factor (eIF-2α) by the double-stranded RNA-activated inhibitor (DAI) kinase correlates with inhibition of translation initiation. The importance of eIF-2α phosphorylation in regulating translation was studied by expression of specific mutants of eIF-2α in COS-1 cells. DNA transfection of certain plasmids could activate DAI kinase and result in poor translation of plasmid-derived mRNAs. In these cases, translation of the plasmid-derived mRNAs was improved by the presence of DAI kinase inhibitors or by the presence of a nonphosphorylatable mutant (serine to alanine) of eIF-2α. The improved translation mediated by expression of the nonphosphorylatable eIF-2α mutant was specific to plasmid-derived mRNA and did not affect global mRNA translation. Expression of a serine-to-aspartic acid mutant eIF-2α, created to mimic the phosphorylated serine, inhibited translation of the mRNAs derived from the transfected plasmid. These results substantiate the hypothesis that DAI kinase activation reduces translation initiation through phosphorylation of eIF-2α and reinforce the importance of phosphorylation of eIF-2α as a way to control initiation of translation in intact cells.

Polypeptide chain synthesis is initiated when eucaryotic initiation factor 2 (eIF-2), GTP, and initiator Met-tRNA form a ternary complex which binds to the 40S ribosomal subunit to generate a 43S preinitiation complex. The 43S preinitiation complex binds mRNA, and subsequently the 60S ribosomal subunit joins to form the 80S initiation complex, with the concomitant hydrolysis of GTP to GDP. To reinitiate, GDP bound to eIF-2 must be exchanged for GTP, a reaction catalyzed by the guanine nucleotide exchange factor (GEF) (32; also identified as eIF-2B [24]). Phosphorylation of the alpha subunit of eIF-2 [eIF-2α) is thought to stabilize the GEF–eIF-2–GDP complex and consequently prevent GTP exchange and eIF-2 recycling (for reviews, see references 28, 31, and 35).

Two protein kinases have been characterized which phosphorylate eIF-2α. The hemin-controlled repressor (HCR) kinase has been studied in reticulocytes and is activated by various stimuli, including hemin deprivation and heat treatment. The double-stranded RNA-activated inhibitor (DAI) kinase is induced by interferon, and its activity is dependent on double-stranded RNA. Upon viral infection, induction and activation of DAI kinase occurs as part of the host antiviral response. Several viruses encode specific gene products which can circumvent the translation inhibition imposed by activation of these kinases (43). Adenovirus has a specific gene product transcribed by RNA polymerase III, the adenovirus-associated (VA) RNA, which inhibits DAI kinase activation and circumvents the host antiviral response (17, 23, 30, 36, 42, 46). Adenovirus deletion mutants in the VA gene (49) produce functional viral mRNAs which are not translated as a result of DAI kinase activation, believed to be mediated by double-stranded RNA derived from transcription off both strands of the viral genome (26, 34).

In a similar manner, transient transfection of certain plasmid DNAs may also activate DAI kinase, although the specific mechanism has not been identified. Under these circumstances, the mRNA expressed from plasmid DNA is inefficiently translated, and adenovirus VA RNA can potentiate translation of the plasmid-derived mRNAs (18, 47, 48). In this case, VA RNA likely promotes translation by preventing activation of the DAI kinase (1, 20). The importance of DAI kinase activation in suppressing translation is also suggested by a similar translation enhancement which occurs upon addition of the DAI kinase inhibitor 2-aminopurine to the conditioned medium of transfected cells. The translation stimulation mediated by both VA RNA and 2-aminopurine is restricted to the plasmid-derived mRNAs, since there is no quantitative or qualitative effect on global protein synthesis (20). One hypothesis to account for this specific effect is that transcription from both strands of the plasmid DNA yields mRNAs which have a partially double-stranded feature to mediate DAI kinase binding to the mRNA, resulting in kinase activation in the vicinity of the mRNA (5, 20). Data from in vitro experimental systems suggest that activation of HCR and DAI kinase likely mediates translational suppression through phosphorylation of eIF-2α. In this report, we demonstrate that suppressed translation is a result of eIF-2 phosphorylation and that the translational enhancement by VA RNA results from its ability to block eIF-2 phosphorylation.

Like other protein complexes involved in guanine nucleotide exchange (35), eIF-2 consists of three nonidentical subunits: α (36 kilodaltons [kDa]), β (38 kDa), and γ (52 kDa). Cloning of the human eIF-2α cDNA (15) permitted identification of the amino acid residue phosphorylated by eIF-2α kinases. Site-directed mutagenesis of eIF-2α cDNA and expression of the mutant forms in an SP6 in vitro transcription-reticulocyte lysate translation system demonstrated that the serine at residue 51 is the most likely site of phosphorylation by HCR and DAI kinases (33). However, previous reports based on in vitro phosphorylation of puri-

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fied eIF-2 had identified either serine 51 (4) or serine 48 (52) as the site of phosphorylation. To ascertain the physiological importance of these two serines, we constructed and studied the effects of expression of eIF-2α genes harboring mutations at both amino acid residues, 51 and 48. We analyzed whether either the expressed wild-type or mutant forms of eIF-2α could antagonize DAI kinase action and therefore alleviate the VA RNA requirement for efficient translation of mRNAs derived from plasmid DNA after transient transfection of COS-1 cells. The results demonstrated that increased expression of the wild-type eIF-2α did not affect translation of mRNAs derived from heterologous plasmids. However, expression of serine-to-alanine mutants at either residue 48 or residue 51 could substitute for VA RNA to promote translation of plasmid-derived mRNAs. Finally, expression of a mutant eIF-2α that had an aspartic acid at amino acid residue 51, created to mimic the charge of the phosphorylated serine, was very poorly translated in vivo, and its expression reduces translation of mRNA from a cotransfected plasmid.

MATERIALS AND METHODS

Plasmid constructions. (i) Construction of the eIF-2α expression plasmid. The plasmids used for eIF-2α expression were pMT2 (2) and pMT2VA$^-$. These plasmids were derived by introducing the transcription unit from the tetracycline-resistant pBR322-based p91023 vector (53) into derivatives of pUC18. In a clockwise orientation (see Fig. 1), pMT2 and pMT2VA$^-$ both contain the simian virus (SV40) origin of replication and transcriptional enhancer (*Pvu*II [0.712 map unit {m.u.} to *Hin*dIII [0.646 m.u.]), the adenovirus major late promoter (*Xho*I [15.8 m.u.] to *Hin*dIII [17.1 m.u.]), the majority of the tripartite leader present on adenovirus late mRNAs (a cDNA copy extending from the 5′ end to the *Xho*I site [26.55 m.u.] in the third leader), a hybrid intron from the 5′ splice site of the first late leader of adenovirus mRNA and a 3′ splice site from an immunoglobulin gene (22), a dihydrofolate reductase (DHFR)-coding sequence (22) present in the 3′ end of the transcript, and the SV40 early polyadenylation signal (*Bcl*I [0.188 m.u.] to *Bam*HI [0.143 m.u.]). They contain the beta-lactamase gene, the colicin E1 origin of replication, and a unique *Eco*RI cloning site for insertion of cDNA clones. These plasmids are identical except that pMT2 contains the adenovirus VAI gene (*Hpa*I [28.02 m.u.] to *Bal*I [29.62 m.u.]) downstream of the SV40 polyadenylation site. The eIF-2α cDNA was excised from pSP65-2alpha (15) by digestion with *Hin*cII, addition of *Eco*RI linkers, and digestion with *Eco*RI. The 1.6-kilobase-pair *Eco*RI fragment was isolated after agarose gel electrophoresis and ligated to pMT2VA$^-$ and pMT2, which had previously been digested with *Eco*RI and treated with calf intestine phosphatase. This 1.6-kilobase-pair cDNA fragment contains 70 base pairs of 5′ untranslated sequence and 604 base pairs of 3′ untranslated sequence. The ligated DNA was transformed into *Escherichia coli* DH5 and plated onto bacterial plates containing 50 μg of ampicillin per ml. DNA was prepared from transformants and screened for the presence of the eIF-2α sequence in the correct orientation by restriction endonuclease digestion and gel electrophoresis. Two clones were obtained, peIF-2αVA$^+$ and peIF-2αVA$^-$.

(ii) Mutagenesis of eIF-2α. The derivation of the 51 alanine mutant (51 A) has been described previously (33). The eIF-2α fragment encoding the 51 alanine mutant was introduced into pMT2VA$^-$ and pMT2 to obtain p51AVA$^-$ and p51AVA$^+$, respectively. Oligonucleotide-directed mutagenesis was performed to change the serine at amino acid residue 48 to an alanine (48 A) or an aspartic acid (48 D) and the serine at residue 51 to an aspartic acid (51 D). Three oligonucleotides of the sequences (underlined nucleotides denote changes)

48 A: 5′-CGCCTTCTGGATAATTCAGCAAGAAGAATCATGCCTTC-3′
48 D: 5′-GAAGGCATGATTCTTCTTGATGAATTATCCAGAAGGCG-3′
51 D: 5′-GATTCTTCTTAGTGAATTAGATAGAAGGCGTATCCGTTC-3′

were synthesized and used for mutagenesis, using the gapped heteroduplex procedure of Morinaga et al. (27) with modifications. A 10-μg amount of peIF-2αVA$^-$ was digested with *Nde*I to linearize the plasmid outside the eIF-2α sequences and then treated with calf intestine phosphatase. A 10-μg amount of pMT2VA$^-$ was digested with *Eco*RI and similarly treated with calf intestine phosphatase. Each preparation of DNA was electrophoresed on low-temperature-melting agarose gels, and the linear forms were isolated by adsorption to and extraction from silica dioxide. Then 1-μg amounts of the preparations were mixed and denatured in a volume of 20 μl of 0.2 N NaOH at room temperature for 10 min. The mixture was subsequently neutralized with 180 μl of 0.02 N HCl–0.1 M Tris hydrochloride (pH 8.0). A 20-pmol amount of the phosphorylated mutagenic oligonucleotide was added to 40 μl of the heteroduplex mixture, and the mixture placed at 68°C for 90 min. After the incubation, the mixture was slowly cooled to room temperature. Each mutagenesis reaction was adjusted to 2 mM $MgCl_2$, 1 mM beta-mercaptoethanol, 400 μM ATP, a 100 μM concentration of each deoxyribonucleotide triphosphate, 3 to 4 U of Klenow fragment of *E. coli* DNA polymerase I, and 400 Units of T4 DNA ligase. The reactions were incubated for 10 min at room temperature and then transferred to 16°C for incubation overnight. Reactions were terminated by phenol-chloroform (1:1) extraction and precipitated by addition of ethanol. The DNA was then used to transform *E. coli* DH5, and the ampicillin-resistant transformants were screened for hybridization to the following oligonucleotides, which were radiolabeled with T4 polynucleotide kinase and [γ-$^{32}PO_4$]ATP:

48 A: 5′-TCTTCTTGCTGAATTA
48 D: 5′-TCTTCTTGATGAATTA
51 D: 5′-GAATTAGATAGAAGG

Filter hybridizations were performed at 37°C for 48 A and at 38°C for 48 D and 51 D in 5× SSC (SSC is 150 mM NaCl plus 15 mM sodium citrate) with 5× Denhardt reagent, 0.1% sodium dodecyl sulfate (SDS), and 100 μg of salmon sperm DNA per ml for 12 h. Filters were washed in 5× SSC with 0.1% SDS at 37°C and then prepared for autoradiography. Positively hybridizing clones were identified, and DNA was isolated and retransformed into *E. coli* for further analysis. Mutations were confirmed by Southern blot hybridization, using oligonucleotide probes, digestion with frequent-cutting restriction endonucleases, and sequencing by the method of Sanger et al. (41), for which an oligonucleotide (5′-GACAACCACACACTCA-3′) labeled with $^{32}PO_4$ at its 5′ end was used. The eIF-2α expression plasmids harboring the correct changes were p48DVA$^-$, p51DVA$^-$, and p48AVA$^-$. The p48 alanine mutation was then subcloned into the pMT2 vector that contains the adenovirus VAI gene by digestion of p48AVA$^-$ with *Bgl*II and *Hpa*I, isolation of the eIF-2α fragment on a low-melting agarose gel, and ligation to pMT2

DNA previously digested with *Bgl*II and *Hpa*I. The resultant plasmid was p48AVA$^+$.

(iii) Derivation of p91023, pD61, and pMT2ADA′. The two DHFR expression plasmids used in this study were p91023, which contains the adenovirus VAI and VAII genes (*Hpa*I [28.02 m.u.] to *Hin*dIII [31.66 m.u.]), and pD61, which is identical to p91023 but lacks both the VAI and VAII genes. Both plasmids contain the same transcription unit that is present in the pMT2 vectors. The adenosine deaminase (ADA) expression plasmids pMT2ADA′ and pMT2AD A′VA$^-$ were obtained by introduction of the *Eco*RI-*Hpa*I fragment encoding murine ADA from p9ADA (21) into the *Eco*RI and *Hpa*I sites of pMT2′ and pMT2′VA$^-$, respectively. pMT2′ was obtained from pMT2 by deletion of the 5′ untranslated tripartite leader such that the 5′ untranslated region of the mRNA reads 5′-ACUCCACCAUG-3′ (S. S. Jones et al., unpublished observations). This deletion results in an mRNA which is translated in a VA RNA-dependent manner.

Cell culture, DNA transfection, and analysis. (i) Expression of wild-type and mutant forms of eIF-2α in COS-1 cells. Plasmid DNA was transfected into COS-1 cells by the DEAE dextran procedure (29). DNA (2 μg/ml) was prepared in Dulbecco minimal essential medium containing 250 μg of DEAE dextran (molecular weight, 500,000; Pharmacia Fine Chemicals, Piscataway, N.J.)–0.1 M Tris hydrochloride (pH 7.3). COS-1 cells that had been plated 20 h previously were rinsed with serum-free medium and fed 4 ml of medium containing DNA. Cells were incubated for 6 to 12 h at 37°C; the medium was then removed, and 10% dimethyl sulfoxide was added for 2 min. The dimethyl sulfoxide was removed, and complete medium containing 0.1 mM chloroquin was added. After 2 h at 37°C, the chloroquin was removed and Dulbecco minimal essential medium containing 10% fetal calf serum was applied. After 60 to 72 h, cells were labeled with [^{35}S]methionine (100 μCi/ml of 1,134 Ci/mmol; 5 mCi/0.5 ml; Dupont NEN Research Products, Boston, Mass.) in methionine-free minimal essential medium supplemented with glutamine and 2% fetal calf serum. Cell extracts were prepared by lysis, using RIPA buffer (22) containing 1 mM phenylmethylsulfonyl fluoride, and analyzed by electrophoresis on reducing 12% SDS-polyacrylamide gels (25). Immunoprecipitations were performed with a rabbit anti-HeLa eIF-2α antiserum and protein A-Sepharose for immunoadsorption.

(ii) Coexpression of wild-type and mutant forms of eIF-2α with DHFR in the presence and absence of the VA genes. Eight-microgram amounts of the DHFR expression plasmid pD61VA or p91023VA$^+$ and of each of the different eIF-2α expression plasmids were mixed and transfected into COS-1 cells as described above. At 64 h posttransfection or at the time indicated, the cells were labeled with [^{35}S]methionine (100 μCi/ml) for 20 min, and cell extracts were prepared by lysis in RIPA buffer. Total cell extracts were electrophoresed on 12% SDS-polyacrylamide gels, and the gels were prepared for autoradiography by treatment with E^{3}H (Dupont, NEN). Band intensity on autoradiograms was quantitated by using an Ultroscan laser densitometer (model 2202; LKB Instruments, Inc., Rockville, Md.). Protein was quantitated by the method of Bio-Rad Laboratories, Richmond, Calif.). [^{35}S]methionine incorporation was monitored by trichloroacetic acid precipitation.

(iii) Fluorescence-activated cell sorting. Transfected COS-1 cells were stained from 48 to 64 h posttransfection by the addition of 3 μM MTX-F (Molecular Probes, Inc., Eugene, Ore.) to the conditioned medium. The labeled cells were prepared for analysis and fluorescence-activated cell sorting by using an EPICS V cell sorter (Coulter Electronics, Inc., Hialeah, Fla.) as previously described (20).

(iv) mRNA analysis. Total cellular RNA was prepared by the procedure Derman et al. (7). RNA blot hybridization was performed as described previously (51). RNA blots were hybridized to probes from either the DHFR-coding fragment or the eIF-2α-coding fragment prepared by nick translation. Total cell RNA was translated in vitro with reticulocyte lysate (Promega Biotech) as recommended by the supplier.

(v) Phosphorylation state of eIF-2α expressed in COS-1 cells. The phosphorylation state of the eIF-2α expressed in COS-1 cells was monitored by labeling COS-1 transfected cells at 64 h posttransfection with 2 ml of $^{32}PO_4$ (400 μCi) for 4 h. Extracts were prepared and immunoprecipitated with the rabbit anti-HeLa eIF-2α antiserum, using protein A-Sepharose as the immunoadsorbent. Samples were electrophoresed on 12% SDS-polyacrylamide gels. Gels were fixed and prepared for autoradiography.

(vi) In vitro phosphorylation by HCR kinase. The ability of the various forms of eIF-2α to serve as substrates for the HCR kinase was examined by preparing extracts of COS-1 transfected cells at 64 h posttransfection. Cells were lysed in kinase lysis buffer (20 mM Tris hydrochloride [pH 7.6], 100 mM KCl, 2 mM $MgCl_2$, 0.5% Nonidet P-40), and equal portions were taken for in vitro kinase reactions in the presence of added [γ-$^{32}PO_4$]ATP (0.1 mM) and HCR kinase. After 15 min at 30°C, equal portions were electrophoresed on 12% SDS-polyacrylamide gels, and the gels were prepared for autoradiography.

(vii) Two-dimensional polyacrylamide gel electrophoresis. COS-1 cells were harvested 64 h posttransfection and lysed in a solution containing 1.2 g of urea, 680 μl of H_2O, 400 μl of 10% Nonidet P-40, and 100 μl of ampholytes (pH 3 to 10). Two-dimensional analysis was performed as previously described (9).

RESULTS

Requirement of VA RNA for efficient translation of mRNA transcribed from some plasmids. Our initial observation was that transient transfection of plasmid DNA expression vectors can yield mRNA that is poorly translated (18, 20). The translational efficiency of mRNA transcribed from plasmid DNAs containing identical transcription units can vary, depending on the vector backbone (M. V. Davies and R. J. Kaufman, unpublished observations). For this reason, two different sets of expression vectors which yield mRNA from identical transcriptional control elements were used in these studies (Fig. 1). The first vectors, p91023 and pD61, have the pBR322 origin of replication and the tetracycline resistance gene. p91023 has, in addition, the adenovirus VAI and VAII genes. These pBR322-based vectors yield DHFR mRNAs that are poorly translated in the absence of VA RNA. The second vectors, pMT2 and pMT2VA$^-$, contain the pUC18 origin of replication and ampicillin resistance gene and yield DHFR mRNAs that are efficiently translated in the absence of VA RNA. Wild-type and mutant eIF-2α cDNAs were introduced into pMT2 and pMT2VA$^-$.

Expression of wild-type and mutant forms of eIF-2α. To examine the role of eIF-2α phosphorylation in translation of plasmid-derived mRNAs, we constructed serine-to-alanine mutants of eIF-2α at the proposed sites of phosphorylation at amino acid residues 48 and 51. The wild-type and mutant forms of the eIF-2α cDNA were expressed in the pMT2 and pMT2VA$^-$ vectors. Expression of the wild-type and mutant

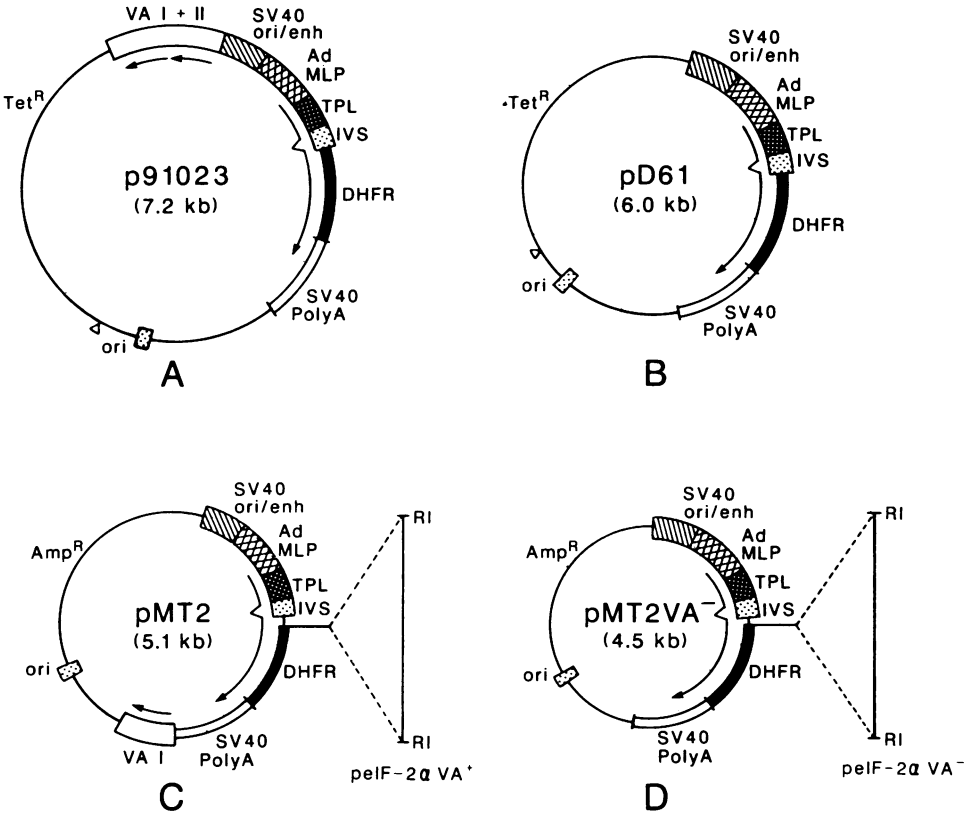

FIG. 1. Expression plasmids used. The DHFR expression plasmids p91023 (A) and pD61 (B) have been described (18, 53). The transcription unit is composed of the SV40 origin and enhancer element, the adenovirus major late promoter containing the majority of the tripartite leader present on adenovirus late mRNAs, an intervening sequence (IVS), a DHFR-coding region, and the SV40 early polyadenylation signal (SV40 PolyA). These plasmids have the tetracycline resistance gene and the pBR322 plasmid backbone. The expression plasmids for eIF-2α expression were pMT2 (C) and pMT2VA$^-$ (D). They have the same transcription unit as do p91023 and pD61 but contain the ampicillin resistance gene and pUC18 plasmid backbone. The eIF-2α cDNA was inserted upstream of the DHFR-coding region in pMT2 and pMT2VA$^-$ to obtain peIF-2αVA$^+$ and peIF-2αVA$^-$. The serine-to-alanine (A) or aspartic acid (D) mutation of eIF-2α were introduced into the eIF-2α-coding region to derive p48AVA$^-$, p51AVA$^-$, p48DVA$^-$, and p51DVA$^-$ in the VAI-deficient plasmids and p48AVA$^+$ and p51AVA$^+$ in the VAI-containing plasmid. For details, see Materials and Methods.

forms of eIF-2α was achieved by transient DNA transfection of COS-1 cells. Synthesis of eIF-2α was monitored by short-pulse labeling of transfected COS-1 cells and analysis of the total cellular protein synthesis by reducing SDS-polyacrylamide gel electrophoresis and fluorography. Figure 2A shows results from COS-1 cells transfected with wild-type eIF-2α plasmids peIF-2αVA$^+$ (lane 2) and peIF-2αVA$^-$ (lane 1) in comparison with the 48 alanine mutant (p48AVA$^-$, lane 3) or with the 51 alanine mutant (lane 4 [p51AVA$^-$] and lane 5 [p51A'VA$^-$], representing independent mutagenized clones) in the pMT2VA$^-$ vector. All plasmids directed the synthesis of a prominent band migrating at approximately 36 kDa (representing approximately 15% of total protein synthesis) which was not present in cells that did not receive DNA (lane 6). The identity of this band as eIF-2α was determined by immunoprecipitation with a rabbit anti-human polyclonal antiserum specific to eIF-2α (see Fig. 6A, lanes 1 through 4). The presence of VA RNA did not increase expression of either the wild-type (Fig. 2A, lanes 1 and 2) or the 48 and 51 serine-to-alanine mutant forms of eIF-2α (data not shown) when expressed in the pMT2 vector, since mRNA from pMT2 vectors is not affected by the presence of VA RNA.

The levels of eIF-2α mRNA produced from parallel transfections with the eIF-2α expression plasmids were determined by Northern (RNA) blot hybridization of total cell RNA isolated from transfected cells, using an eIF-2α (data not shown) or a DHFR (Fig. 2B) probe. Since the 3′ end of the eIF-2 transcript contained DHFR sequences (Fig. 1), it hybridized to a DHFR probe. All plasmids produced similar levels of an eIF-2α mRNA (Fig. 2B). Therefore, the similar levels of eIF-2α synthesis for the wild-type and alanine mutant forms (Fig. 2A) correlated with similar levels of mRNAs expressed from the different pMT2-based vectors. In contrast, when the eIF-2α cDNA was expressed in the pBR322-based vectors, the resultant eIF-2α mRNA required the presence of VA RNA for efficient translation (data not shown).

In vitro and in vivo phosphorylation of eIF-2α expressed in COS-1 cells. The ability of the wild-type and mutant forms of eIF-2α to serve as substrates for phosphorylation by HCR kinase was monitored by in vitro phosphorylation reactions. Transfected COS-1 cells were harvested, and lysates were prepared and analyzed for ability of the expressed eIF-2α to serve as a phosphorylation substrate for exogenously added HCR kinase. The wild-type eIF-2α in the presence (Fig. 3A, lane 5) or absence (Fig. 3A, lane 6) of VA RNA and the 48 serine-to-alanine mutant in the absence of VA RNA (Fig. 3A, lane 4) could serve equally well as substrates for HCR kinase. In contrast, no detectable phosphate was incorporated into the 51 alanine form of eIF-2α (Fig. 3A, lane 3). All reactions exhibited similar levels of HCR autophosphoryla-

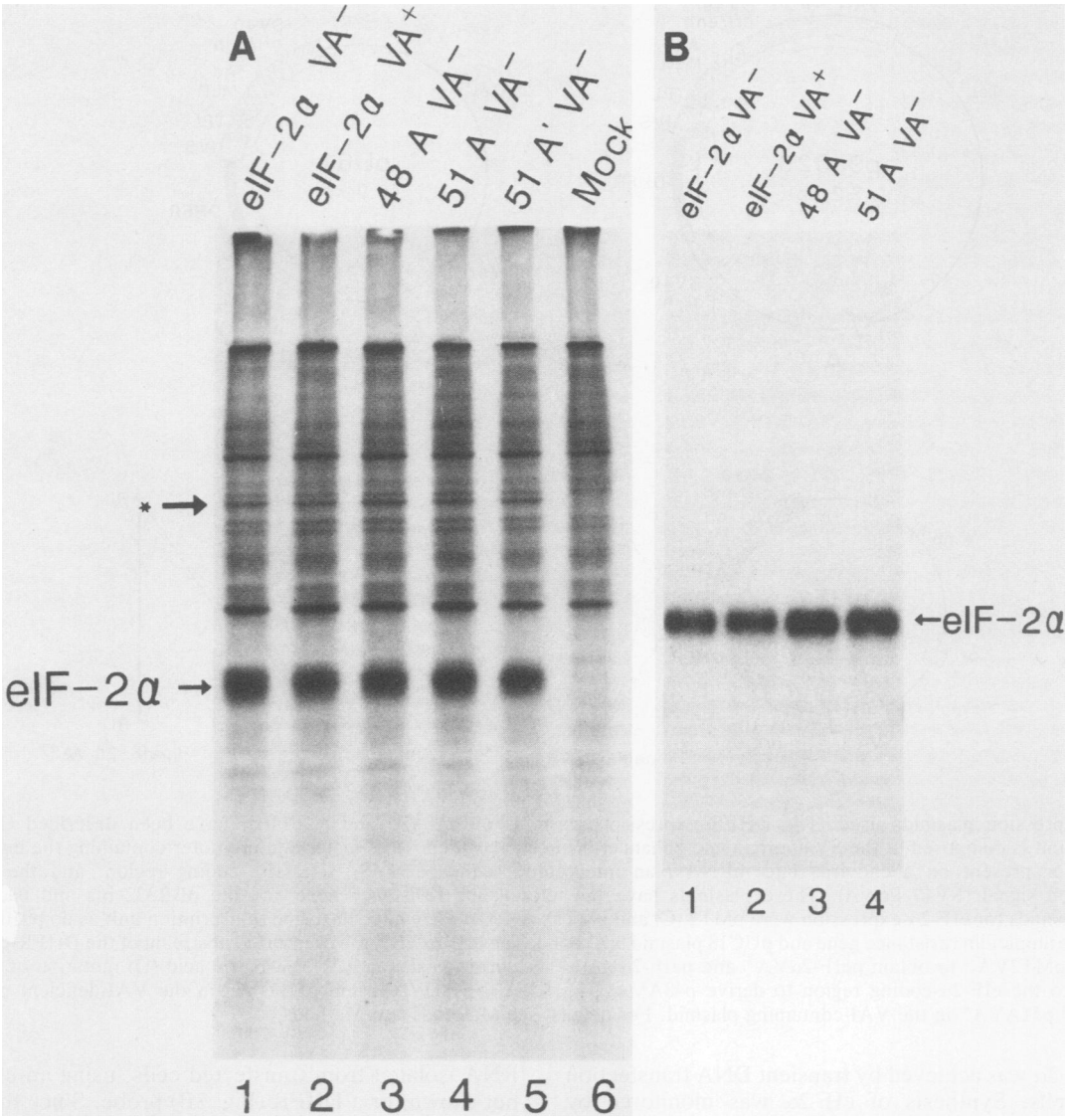

FIG. 2. Expression of wild-type and alanine mutant forms of eIF-2α. Plasmid DNA was transfected into COS-1 cells, and expression was analyzed by [^{35}S]methionine labeling for 20 min at 64 h posttransfection and electrophoresis of equal amounts of total cell protein (A). In parallel, total RNA was isolated and analyzed by RNA blot hybridization to a DHFR-specific probe (B). Similar results were obtained by hybridization to an eIF-2α-coding-region probe. The lanes are marked with the transfected plasmid encoding the wild type (eIF-2α) or the 48 or 51 alanine mutant (48A or 51A) in the presence (VA^+) or absence (VA^-) of the adenovirus VAI gene. Positions of the eIF-2α protein and mRNA are shown. The asterisk indicates the heat shock protein of 70 kDa which is induced by eIF-2α overexpression.

tion, which provided an internal control for the activity of the enzyme in these reactions and demonstrated that eIF-2α expressed in the cell extract did not inhibit the kinase. These results are consistent with the findings of Pathak et al. (33), which demonstrate the serine at amino acid residue 51 is essential for HCR-mediated phosphorylation.

The in vivo phosphorylation state of the expressed eIF-2α was studied by labeling transfected COS-1 cells for 4 h with $^{32}PO_4$. For this experiment, the VA-deficient expression plasmid encoding the wild-type or mutant form of eIF-2α was cotransfected with a DHFR expression plasmid that contains (p91023) or lacks (pD61) the adenovirus VAI and VAII genes. Cell prepared and analyzed by immunoprecipitation with a rabbit-anti human eIF-2α antibody exhibited two phosphoprotein forms which migrated between 36 and 40 kDa (Fig. 3B, bands I and II). The primary phosphorylated species (band I) represented eIF-2α in cells transfected with the wild type or the 48 alanine mutant (Fig. 3B, lanes 7 and 8, respectively) in the absence of VA RNA. Severalfold-reduced phosphate incorporation was observed in the wild-type eIF-2α and 48 alanine mutant when VA RNA was provided by cotransfection with a plasmid containing the VA genes (Fig. 3B, lanes 2 and 3, respectively). The reduction in phosphate incorporation into the wild type or the 48 alanine mutant in the presence of VA RNA suggested that DAI kinase was responsible for the in vivo phosphorylation. Expression of the 51 alanine mutant exhibited significantly less of the DAI-mediated phosphorylated species in both the presence (Fig. 3B, lane 4) and absence (Fig. 3B, lane 9) of VA RNA provided by the cotransfected plasmid. The low level of phosphate incorporation observed may represent phosphorylation of the endogenous eIF-2α. In addition to

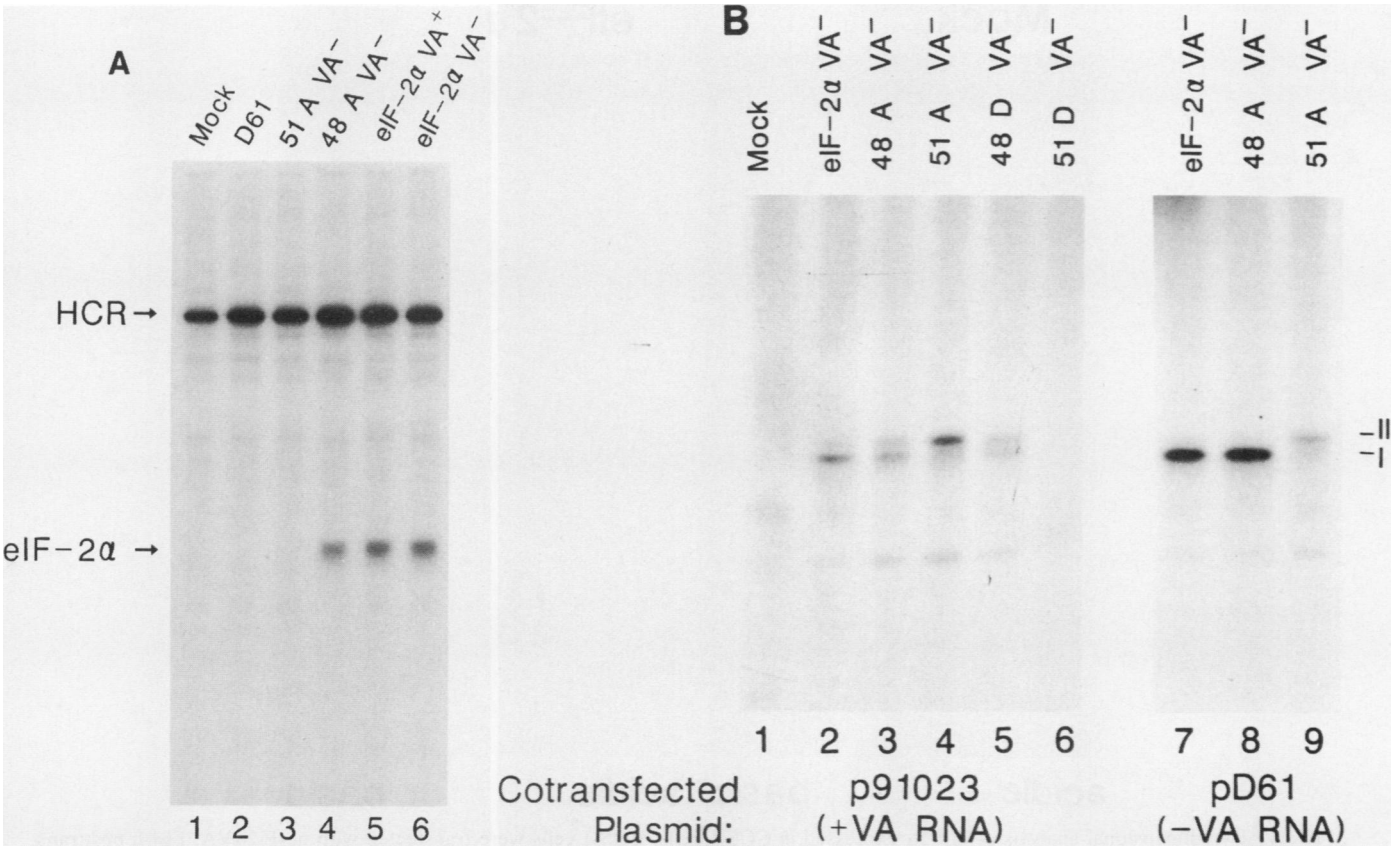

FIG. 3. In vitro and in vivo phosphorylation of wild-type and mutant forms of eIF-2α. (A) COS-1 cells were transfected with the indicated eIF-2α expression vectors; cell extracts were prepared at 64 h posttransfection and incubated in the presence of [γ-^{32}P]ATP and HCR kinase. After 15 min, samples were electrophoresed on a reducing SDS-polyacrylamide gel. Migration of the phosphorylated eIF-2α and HCR kinase is indicated. The mock cells-infected did not receive plasmid DNA. (B) COS-1 cells were cotransfected with the indicated eIF-2α expression plasmid and a plasmid either containing (p91023) or lacking (pD61) the adenovirus VAI and VAII genes. Cells were labeled at 64 h posttransfection with $^{32}PO_4$ for 4 h, and extracts were prepared and immunoprecipitated with an anti-eIF-2α antiserum. Immunoprecipitates were analyzed by electrophoresis on a reducing SDS-polyacrylamide gel. Band I indicates the mobility of the DAI-mediated phosphorylated species of eIF-2α; the identity of band II is not known.

the eIF-2α phosphorylated species, there was a slower-migrating species (Fig. 3B, band II), which was especially predominant in cells transfected with the 51 alanine mutant in the presence of VA RNA (Fig. 3B, lane 4). We do not know the identity of this species; however, it is interesting to note that its presence correlated with the amount of non-phosphorylated or active eIF-2. These in vivo labeling experiments demonstrate that amino acid residue 51 is an important requirement for in vivo phosphorylation by DAI kinase and that VA RNA can reduce phosphorylation of plasmid-encoded eIF-2α in intact cells.

The steady-state level and phosphorylation pattern of the expressed eIF-2α was monitored by two-dimensional protein blotting procedures (Fig. 4) (9). By comparison of band intensities between mock- and eIF-2α-transfected cells, the level of eIF-2α expression is estimated to be at least 25-fold greater than that in cells that did not receive DNA. From the staining intensity of the two-dimensional gel and comparison with actin (Fig. 4, compare b and c), we estimate that the expression level represents approximately 3 to 5 μg/10^6 cells by assuming that actin represents approximately 4% of the cellular protein. This expression level is also consistent with a 25- to 50-fold overproduction of eIF-2α if eIF-2α represents 0.1% of total cellular protein (9). Comparison of the basic and acidic variant spot intensities shows that the overexpressed eIF-2α (compare a and b in Fig. 4) and the 48 alanine mutant (data not shown) exhibited ratios of phosphorylated to nonphosphorylated forms of eIF-2α similar to that normally observed in growing cells (9). Thus, the kinase(s) and the phosphoprotein phosphatase(s) responsible were not saturated by the increased eIF-2α expression. In contrast, expression of the 51 alanine mutant of eIF-2α resulted in the single basic species which comigrated with unmodified eIF-2α produced in a reticulocyte lysate (data not shown).

Expression of the 48 or 51 alanine mutant of eIF-2α interferes with DAI kinase action. The effect of increased levels of wild-type and mutant eIF-2α on the translation efficiency of plasmid-derived mRNAs was monitored by a cotransfection assay. COS-1 cells were cotransfected with DHFR expression plasmid pD61 or p91023 and with an equal amount of wild-type or mutant eIF-2α expression plasmid DNA or a control plasmid DNA, pMT2ADA′ or pMT2ADA′VA⁻, which encode murine adenosine deaminase (ADA) in the presence and absence of the VAI gene, respectively. The DHFR expression vectors shown in Fig. 1, p91023 and pD61, produced similar amounts of DHFR mRNA from identical elements (20). Efficient translation of the DHFR mRNA required the presence of adenovirus VA RNA, which was supplied within the p91023 vector but not

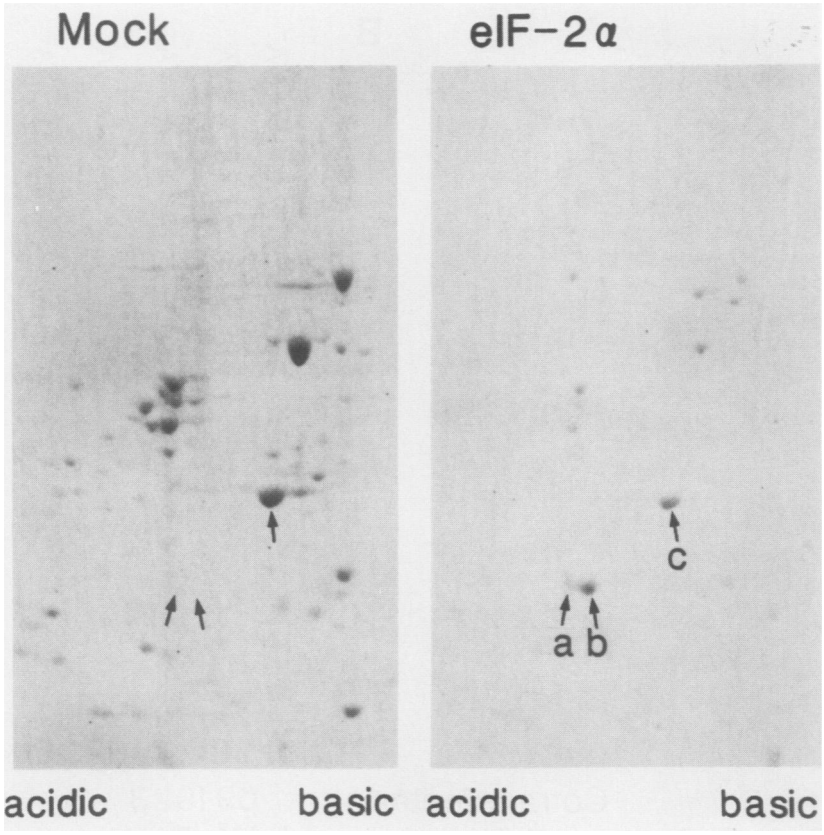

FIG. 4. Two-dimensional analysis of eIF-2α expressed in COS-1 cells. COS-1 cells were transfected with peIF-2αVA$^-$; 64 h posttransfection, total cell lysates prepared for two-dimensional gel electrophoretic analysis by Coomassie blue staining as described in Materials and Methods. The mock-infected control cells received no DNA. The phosphorylated (a) and nonphosphorylated (b) forms are indicated on the right, and their respective positions are identified on the left. The migration of actin is also shown (c). The acidic and basic ends of the gel are indicated. The left panel received 12.5 times less protein in order to compensate for the overexpression of eIF-2α. The level of eIF-2α expression is estimated at greater than 25-fold the endogenous level, and approximately 10 to 30% was phosphorylated.

within pD61 (Fig. 1) (18). Protein synthesis within the transfected subpopulation was studied by staining cells with a fluorescent analog of methotrexate (MTX-F) that quantitatively binds DHFR in living cells and then using a fluorescence-activated cell sorter to isolate the fluorescence-positive cells after [^{35}S]methionine pulse-labeling (19, 20). The fluorescence histograms of cells transfected with either pD61 or p91023 exhibited a 20% subpopulation that stained more brightly with MTX-F than did cells that did not receive DNA (data not shown; 20). The subpopulation of cells transfected with p91023 or cotransfected with pD61 and either the 48 or the 51 alanine mutant of eIF-2α stained brighter than did the subpopulation of cells that received pD61 with the wild-type eIF-2α VA$^-$ or ADA VA$^-$ control expression plasmid (data not shown). Protein synthesis in the entire transfected cell population (Fig. 5A, lanes 1 through 6) and in the subpopulations of cells sorted for MTX-F-positive fluorescence (Fig. 5A, lanes 7 through 11) was analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography of equal amounts of protein from prepared cell extracts. Cells were cotransfected with pD61 and a control plasmid, pMT2ADA′VA$^-$, which produces an mRNA for which efficient translation of ADA is VA RNA dependent. These cotransfected cells expressed lower levels of DHFR and ADA (Fig. 5A, lanes 2 and 8) than did cells transfected with plasmids that also encode adenovirus VA RNA, p91023 and pMT2ADA′ (Fig. 5A, lanes 1 and 7). Cells cotransfected with pD61 and wild-type eIF-2α VA$^-$ did not show any change in DHFR expression (Fig. 5A, compare lanes 2 and 8 with lanes 3 and 9).

In contrast to cotransfection of pD61 with the wild-type eIF-2α VA$^-$ expression vector (Fig. 5A, lanes 3 and 9), cells cotransfected with pD61 and either the 51 (Fig. 5A, lanes 4, 10) or 48 (Fig. 5A, lanes, 5, 11) alanine mutant of eIF-2α exhibited an 8- to 10-fold increase in DHFR synthesis. However, the levels of eIF-2α synthesis for the wild type and alanine mutants were similar (Fig. 5A, lanes 3 through 5 and 9 through 11), probably because they were in pMT2-based vectors. No further increase in DHFR translation was observed when the wild-type and alanine mutant forms of eIF-2α were transfected in the presence of the adenovirus VAI gene (data not shown). Similar increases in translation were observed upon cotransfection of the alanine mutant forms of eIF-2α with an ADA expression plasmid in the absence of VA RNA (data not shown). Thus, the increased expression from the plasmid-encoded mRNA was not specific to DHFR.

The level of mRNA encoding eIF-2α and DHFR in the experiment described above was monitored by Northern blot hybridization to a DHFR-specific probe that will also hybridize to the eIF-2α mRNA. The cotransfected cells exhibited similar levels of DHFR and eIF-2α mRNAs (Fig. 5B, lanes 1 through 3). Therefore, alterations in the level of DHFR mRNA were not responsible for the increased expression of DHFR observed by coexpression of the mutant forms of eIF-2α. These experiments demonstrate that

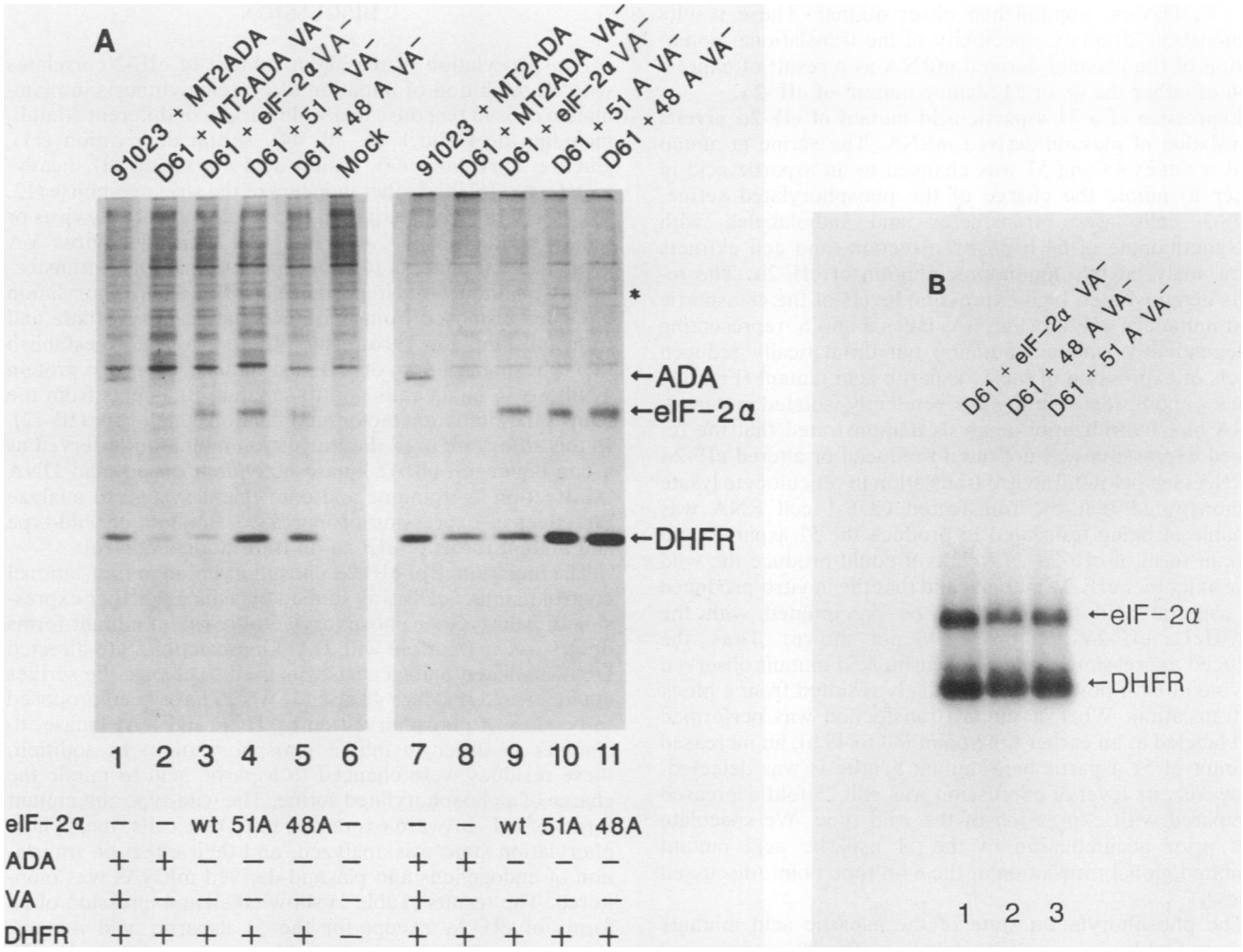

FIG. 5. Effect of wild-type (wt) and mutant eIF-2α expression of protein synthesis. (A) COS-1 cells were cotransfected with either pD61 or p91023 (8 μg each) and an eIF-2α expression plasmid (8 μg) or an ADA expression plasmid (8 μg of pMT2ADA′ or pMT2ADA′VA⁻) and were labeled with MTX-F and [^{35}S]methionine as described in Materials and Methods. Approximately 20% of the cells from each transfection expressed significantly elevated levels of DHFR. Cells that received p91023 or pD61 cotransfected with either the 48 or 51 alanine mutant stained brighter than did cells that received pD61 with either pMT2ADA′VA⁻ or peIF-2αVA⁻. The transfected cells were isolated, and extracts were prepared for protein determination and SDS-polyacrylamide gel electrophoresis. The left panel represents total protein synthesis in the original total cell population; the right panel shows results from the isolated subpopulation of cells that received DNA. Indicated are the position of migration of DHFR, ADA, and eIF-2α. The asterisk represents migration of heat shock protein 70. The presence of the different genes in the transfection are shown below. The plasmid DNAs transfected are shown on top. The fold increase in DHFR translation by the presence of VA RNA shown in lane 1 compared with lane 2 is actually greater than observed when corrected for severalfold-lower DHFR mRNA levels in the cotransfected cells represented in lane 1. (B) Parallel transfected plates were harvested at 64 h posttransfection, and total cellular RNA was isolated for Northern blot hybridization to a DHFR probe as described in Materials and Methods.

increased expression of the wild-type eIF-2α does not influence the translation of DHFR mRNA derived from a cotransfected VA$^+$ or VA$^-$ plasmid. In contrast, expression of either the 48 or the 51 serine-to-alanine mutant of eIF-2α could substitute for the VA requirement to promote efficient DHFR translation. No further translation stimulation was mediated by combining the presence of a mutant form of eIF-2α with the presence of VA RNA (data not shown).

Expression of wild-type or alanine mutant forms of eIF-2α does not alter global mRNA translation. The effect of expression of wild-type or alanine mutant forms of eIF-2α on global mRNA translation was studied by analysis of total protein synthesis in the subpopulation of cells that received plasmid DNA. DHFR was the major protein expressed in the transfected cells that received the DHFR vector either with the VA genes (Fig. 5A, lane 7) or with either the 48 or 51 alanine eIF-2α mutant (Fig. 5A, lanes 11 and 10). Although DHFR synthesis was stimulated 8- to 10-fold by coexpression of the mutant eIF-2α compared with coexpression of the wild-type eIF-2α, there was no detectable quantitative alteration in protein synthesis measured by the specific activity of the cell extracts (counts per minute per microgram of protein) or qualitative alteration in the protein synthesis pattern monitored by one-dimensional gel analysis (Fig. 5A, lanes 7 through 11). One exception was the presence of a 70-kDa protein that appeared in cells that expressed very high levels of eIF-2α. This protein comigrated with the inducible heat shock protein of 70 kDa upon two-dimensional gel analysis

(M. V. Davies, unpublished observations). These results demonstrate dramatic specificity of the translational potentiation of the plasmid-derived mRNA as a result of expression of either the 48 or 51 alanine mutant of eIF-2α.

Expression of a 51 aspartic acid mutant of eIF-2α arrests translation of plasmid-derived mRNA. The serine at amino acid residues 48 and 51 was changed to an aspartic acid in order to mimic the charge of the phosphorylated serine. COS-1 cells were transfected and radiolabeled with [^{35}S]methionine at 64 h posttransfection, and cell extracts were analyzed by immunoprecipitation of eIF-2α. The results demonstrated high expression levels of the 48 aspartic acid mutant of eIF-2α (Fig. 6A, lanes 5 and 6, representing independently isolated mutants) but dramatically reduced levels of expression of the 51 aspartic acid mutant (Fig. 6A, lanes 7 and 8, representing independently isolated mutants). RNA blot hybridization analysis demonstrated that the reduced expression was not due to reduced or altered eIF-2α mRNA (see below). In vitro translation in reticulocyte lysate demonstrated that the transfected COS-1 cell RNA was capable of being translated to produce the 51 aspartic acid mutant form of eIF-2α as well as it could produce the wild type and other eIF-2α mutants and that the in vitro-produced 51 aspartic acid mutant could be precipitated with the anti-HeLa eIF-2α antibody (data not shown). Thus, the reduced expression of the 51 aspartic acid mutant observed in vivo at 64 h posttransfection likely resulted from a block in translation. When a similar transfection was performed and labeled at an earlier time point (40 to 48 h), an increased amount of 51 aspartic acid mutant synthesis was detected. However, its level of expression was still 25-fold decreased compared with expression of the wild type. We speculate that prior accumulation of the 51 aspartic acid mutant inhibited global translation at the 64-h time point (discussed below).

The phosphorylation state of the aspartic acid mutants was studied by in vivo $^{32}PO_4$ labeling. The 48 aspartic acid mutant exhibited phosphorylated species (Fig. 3, lane 5) very similar to the wild-type eIF-2α (Fig. 3, lane 2). It was not possible to detect phosphate incorporation into the 51 aspartic acid form (Fig. 3, lane 6) because of its reduced synthesis.

The effect of the 51 aspartic acid mutant on translation of another mRNA was examined by cotransfection with the DHFR expression plasmid p91023 in the presence of the adenovirus VA RNA genes. The results demonstrated reduced DHFR expression by cotransfection with the 51 aspartic acid mutant (Fig. 6B, lane 3) compared with cotransfection with the 51 alanine mutant (Fig. 6B, lane 2). RNA blot hybridization demonstrated that at least as much DHFR mRNA was present in cells cotransected with the 51 aspartic acid mutant as was present in cells cotransfected with the 51 alanine mutant (Fig. 6B, lanes 4 through 6). Translation in reticulocyte lysate demonstrated that the expressed DHFR mRNAs were translatable with similar efficiencies in vitro (Fig. 6B, lanes 7 through 9). Cotransfection of p91023 with equal amounts of the 51 aspartic acid and 51 alanine mutants of eIF-2α did not improve the level of DHFR translation compared with cotransfection of p91023 with the 51 aspartic acid mutant alone (data not shown). These results show that the 51 aspartic acid mutant of eIF-2α can arrest translation of mRNA derived from a cotransfected plasmid and that the effect observed with the aspartic acid mutant is dominant over the effect observed with the alanine mutant.

DISCUSSION

Phosphorylation of the alpha subunit of eIF-2 correlates with an inhibition of initiation of protein synthesis in mammalian cells in response to a wide variety of different stimuli, including heat shock (6, 10, 44), serum deprivation (11), glucose starvation (44), amino acid starvation (3), heavy-metal ions (16), and other inducers of the stress response (12, 13), interferon treatment with encephalomyocarditis virus or reovirus infection (37, 40), infection with adenovirus VA gene deletion mutants (42, 46), and plasmid DNA transfection (1). Although the importance of eIF-2α phosphorylation has been proposed from these correlative in vivo data and from substantial in vitro results, there is a need to establish that phosphorylation of eIF-2α actually controls protein synthesis in intact cells and to separate its effects from the many other initiation factor modifications that occur (10–12). In this study, we used the translation inhibition observed as a consequence of DAI kinase activation on plasmid DNA transfection as a unique and convenient system to analyze the effect of expression of increased amounts of wild-type and mutant forms of eIF-2α on translational control.

The importance of eIF-2α phosphorylation in translational control in intact cells was studied by asking whether expression of wild-type or phosphorylation-resistant mutant forms of eIF-2α can interfere with DAI kinase action. Site-directed DNA-mediated mutagenesis was used to change the serines at amino acid residues 48 and 51, which have been proposed to be sites of phosphorylation by HCR and DAI kinase, to alanines in order to inhibit phosphorylation. In addition, these residues were changed to aspartic acid to mimic the charge of a phosphorylated serine. The wild-type and mutant forms of eIF-2α were expressed in COS-1 cells, their phosphorylation state was analyzed, and their effect on translation of endogenous and plasmid-derived mRNAs was monitored. The results (Table 1) show efficient expression of all forms of eIF-2α except for the 51 aspartic acid mutant. Expression of the 51 aspartic acid mutant was blocked at the level of translation in vivo. Analysis of the phosphorylation state of the expressed eIF-2α demonstrated that the serine at residue 51 was required for phosphorylation by HCR and DAI kinase. This conclusion is consistent with the proposed phosphorylation site by direct sequencing of eIF-2α (4) and with data from in vitro expression of wild-type and mutant forms of eIF-2α (33).

The ability of the wild-type and mutant forms of eIF-2α to antagonize the action of activated DAI kinase was monitored by DHFR translation from an mRNA transcribed from a cotransfected VA-deficient plasmid. Expression of wild-type eIF-2α did not affect translation of DHFR, whereas expression of either the 48 or 51 alanine mutant of eIF-2α increased DHFR translation to a level similar to that observed by the presence of adenovirus VA RNA. Similar observations were also made upon analysis of another cDNA, ADA, contained within the expression plasmid. Under the conditions of our COS-1 cell transfection, the alanine mutants of eIF-2α did not exert any effect when monitored in the presence of VA RNA. It will be interesting to determine whether the alanine mutants alter the translational response to other types of stress. We conclude that expression of either serine-to-alanine mutant can substitute for the requirement of VA RNA to elicit efficient translation of the plasmid-derived mRNA.

Expression of a serine-to-aspartic acid mutant at residue 48, created to mimic the charge of a phosphorylated serine, did not alter the phosphorylation pattern or protein synthe-

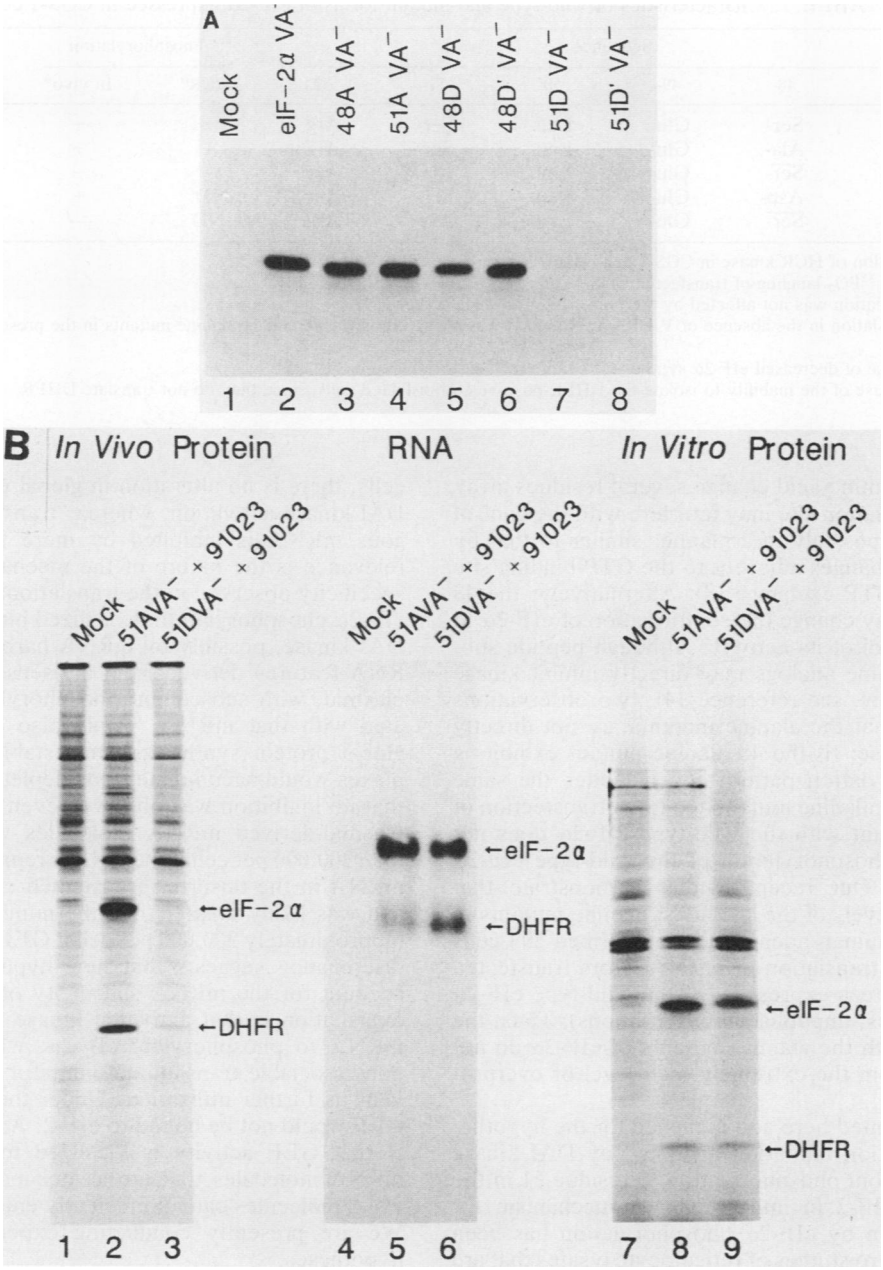

FIG. 6. (A) Expression of aspartic acid mutants of eIF-2α. COS-1 cells were transfected with the indicated eIF-2α expression plasmid DNAs and labeled with [^{35}S]methionine at 64 h posttransfection. Extracts were prepared for analysis by immunoprecipitation with anti-human eIF-2α rabbit antiserum and SDS-polyacrylamide gel electrophoresis. The plasmids used in the transfection are indicated above. Lanes 5 and 6 and lanes 7 and 8 represent independent plasmid mutant isolates of the 48 and 51 aspartic acid mutant forms. (B) Effect of 51 aspartic acid mutant eIF-2α on DHFR translation. COS-1 cells were cotransfected with the 51 alanine mutant or the 51 aspartic acid mutant of eIF-2α and an equal amount of the DHFR expression plasmid p91023. At 40 h posttransfection, the cells were labeled for 20 min with [^{35}S]methionine, and cell lysates were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (lanes 1 through 3). Also at 40 h, total RNA was prepared from parallel plates and analyzed by Northern blot analysis by hybridization to a DHFR-labeled probe (lanes 4 through 6). The isolated RNA was translated in vitro in a rabbit reticulocyte lysate and also analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (lanes 7 through 9). Positions of migration of eIF-2α and DHFR are indicated.

sis. This finding suggests that the addition of charge similar to that of phosphorylated serine at this site does not affect eIF-2 activity. In contrast, eIF-2α with a serine-to-aspartic acid change at residue 51 was expressed poorly as a result of a block in translation. Cotransfection of this mutant with a DHFR expression plasmid resulted in reduced translation of the DHFR mRNA.

We expect that the effects mediated by the alanine and aspartic acid mutants are mediated directly by interference with eIF-2 function and that they reflect the importance of eIF-2α phosphorylation in control of translation initiation. It was unexpected that the 48 alanine mutant would exhibit a phenotype similar to that of the 51 alanine mutant, since the former serves as a substrate for eIF-2α kinases. However, it

TABLE 1. Characteristics of wild-type and mutant forms of eIF-2α expressed in COS-1 cells

eIF-2α	Sequence						Phosphorylation		Translation effect	
	47-	48-	49-	50-	51-	52	HCR[a]	In vivo[b]	Global[c]	DHFR[d]
Wild type	Leu-	Ser-	Glu-	Leu-	Ser-	Arg	+	+	–	–
48 Ala	Leu-	Ala-	Glu-	Leu-	Ser-	Arg	+	+	–	↑
51 Ala	Leu-	Ser-	Glu-	Leu-	Ala-	Arg	–	–	–	↑
48 Asp	Leu-	Asp-	Glu-	Leu-	Ser-	Arg	ND[e]	+	–	–
51 Asp	Leu-	Ser-	Glu-	Leu-	Asp-	Arg	ND	—[f]	ND[g]	↓

[a] Monitored by incubation of HCR kinase in COS-1 cell lysates in the presence of [γ-^{32}P]ATP.
[b] Monitored by in vivo $^{32}PO_4$ labeling of transfected COS-1 cells.
[c] Effect on global translation was not affected by the presence or absence of VA RNA.
[d] Effect of DHFR translation in the absence of VA RNA. No effect was observed by the 48 and 51 alanine mutants in the presence of VA RNA.
[e] ND, Not determined.
[f] Not detectable because of decreased eIF-2α synthesis.
[g] Not determined because of the inability to isolate the DHFR-positive cotransfected cells, since they do not translate DHFR.

is possible that an amino acid change several residues away from the phosphorylation site may interfere with the control of eIF-2 recycling, possibly in a manner similar to that by which amino acid changes adjacent to the GTP-binding site in c-*ras* can alter GTP exchange (8). Alternatively, the 48 alanine mutation may change the conformation of eIF-2α to interfere with control of its activity. Although peptide substrate serine-to-alanine analogs may directly inhibit kinase activity (for a review, see reference 14), two observations cause us to think that the alanine mutants are not directly inhibiting DAI kinase: (i) the 48 alanine mutant exhibits a wild-type phosphorylation pattern and mediates the same effect as does the 51 alanine mutant and (ii) cotransfection of the 51 alanine mutant with the wild-type eIF-2α does not result in reduced phosphorylation of the wild-type eIF-2α (data not shown). Our recent studies demonstrate that expression of low levels of the 48 and 51 alanine mutants in stably transfected human adenovirus-transformed 293 cells can also potentiate translation of mRNAs from transfected plasmid DNA, whereas expression of the wild-type eIF-2α has no effect (Davies, unpublished observations). Thus, the effects observed with the alanine mutants of eIF-2α do not appear to result from the extremely high level of overproduction of eIF-2α.

The results presented here add evidence for the hypothesis that the translation inhibition mediated by DAI kinase activation results from phosphorylation at residue 51 in the alpha subunit of eIF-2 in intact cells. A mechanism for translation inhibition by eIF-2α phosphorylation has been proposed from in vitro studies of reticulocyte lysates that are deprived of heme (31, 35, 39). A critical step in the recycling of eIF-2 involves exchange of GDP for GTP, a reaction catalyzed by GEF. Phosphorylated eIF-2 retains its ability to bind GEF; under these circumstances, however, it cannot promote guanine nucleotide exchange (24, 38, 45). Estimates of the amount of GEF present in the cell range from 20 to 5% of the level of eIF2 (50). Since inhibition of initiation can occur at moderate levels of eIF-2 phosphorylation (phosphorylation of only 30 to 40% of eIF-2), it has been proposed that phosphorylation of the alpha subunit of eIF-2 blocks eIF2 recycling by sequestering limiting amounts of GEF (28, 39).

Our results substantiate the importance of phosphorylation of eIF-2 as a major control in translation of virus- and plasmid-derived mRNAs in intact cells. However, simple sequestering of GEF does not account for the specificity of the translation inhibition observed in transfected cells in the absence of VA RNA. In the subpopulation of transfected cells, there is no alteration in global protein synthesis upon DAI kinase activation, whereas translation of the heterologous mRNA is inhibited by more than 90%. Of critical relevance is the nature of the mechanism allowing for the specificity observed in the translational control mediated by eIF-2α phosphorylation. Localized binding and activation of DAI kinase, possibly by mRNA harboring double-stranded RNA features derived from antisense transcripts from the plasmid, with subsequent phosphorylation of eIF-2 associated with that mRNA, would also be expected to affect global protein synthesis, since stable eIF-2αP-GEF complexes would accumulate, thus depleting GEF. It is striking that no inhibition was observed even though the number of plasmid-derived mRNA molecules was very large (more than 200,000 per cell if the mRNA represents 20% of the total mRNA in the unsorted transfected cell population; Fig. 5) and was likely greater than the number of GEF molecules (approximately 200,000 per cell if GEF is 10% of eIF-2). This discrepancy suggests that new hypotheses are needed to account for the mRNA specificity observed. One possible explanation is that activated kinase may directly bind the mRNA to phosphorylate eIF-2α, which can then form a nondissociable translational inhibitor on the mRNA to prevent its further utilization. Under the inhibited conditions, GEF would not be bound to eIF-2. An alternate explanation is that GEF activity is localized to polysomes and that mRNA molecules that are associated with phosphorylated eIF-2 molecules cannot efficiently enter the polysomal pool. We are presently conducting experiments to test these hypotheses.

The use of site-directed mutagenesis and expression to produce a serine-to-alanine mutant of eIF-2α that cannot serve as a substrate for phosphorylation or to produce a serine-to-aspartic acid mutant that can mimic the charge of the phosphorylated state has provided a means of dissecting the role of eIF-2α phosphorylation in intact cells. Extension of this approach to other phosphoproteins, such as G proteins involved in GTP binding, will provide a method for ascertaining which phosphorylation reactions and substrates have physiological significance.

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