The role of eukaryotic initiation factor 2 (eIF-2) phosphorylation in translational control has been demonstrated in vivo by overexpressing variant forms of eIF-2α that are not phosphorylated. COS-1 cells transiently transfected with expression vectors for human eIF-2α contain 10-20-fold more eIF-2α subunit than the endogenous COS cell eIF-2 trimeric complex. Examination of the variant form of eIF-2α, Ser51Ala, where Asp replaces Ser51, causes inhibition of protein synthesis, whereas the Ser48Asp variant does not. When either Ser48 or Ser51 is replaced by Ala, the variants stimulate dihydrofolate reductase synthesis when the eIF-2α kinase, DAI, is activated. In order to elucidate these mechanisms, we have separated eIF-2 trimeric complexes from free overexpressed eIF-2α subunits by fast protein liquid chromatography Superose chromatography. Pulse-labeled cells transfected with wild-type or variant DNAs produced eIF-2α preparations with greater than 10-fold higher specific radioactivity in the α-subunit compared to the γ-subunit, thus demonstrating that the human eIF-2α produced from the plasmids readily exchanges into COS cell eIF-2 complexes. Both wild-type and Ser48Ala variant forms of the free 2a-subunit, further purified by MonoQ chromatography, are poor substrates for the eIF-2α kinase. None of the purified free eIF-2α subunits inhibits phosphorylation of eIF-2 in vitro, even at up to 8-fold molar excess. Examination of the extent of eIF-2α phosphorylation in the COS cell eIF-2 complexes by two-dimensional polyacrylamide gel electrophoresis shows that the stimulation of dihydrofolate reductase synthesis by the Ser51Ala variant is most readily explained by failure of eIF-2 to be phosphorylated. Stimulation by the Ser48Ala variant appears to occur by mitigation of the effect of phosphorylation at Ser51 since the double variant, Ser48Ala-Ser51Asp, inhibits protein synthesis less than the single variant Ser51Asp. The evidence argues strongly against there being a second site of phosphorylation involved in translational repression.

Eukaryotic initiation factor eIF2-2 is identified as one of the factors involved in regulating protein synthesis (for recent reviews, see Refs. 1, 2). The factor forms a ternary complex with the initiator Met-tRNA, and GTP, and promotes the binding of the initiator tRNA to ribosomes. eIF-2 comprises three dissimilar subunits named α, β, and γ, whose molecular masses are 36, 38, and 52 kDa, respectively. Phosphorylation of the α-subunit of eIF-2 correlates with an inhibition of initiation of protein synthesis and has been implicated in numerous translational control mechanisms (3). Two protein kinases have been characterized that phosphorylate eIF-2α: the hemin-regulated inhibitor (HRI) and the double-stranded RNA-activated inhibitor (DAI). A correlation of eIF-2α phosphorylation and inhibition of protein synthesis has been made in cells subjected to serum deprivation (4), heat shock (5), interferon treatment followed by virus infection (6), transfection with certain plasmid DNAs (7), inter al. It is believed that limited phosphorylation of eIF-2α results in effectively sequestering the less abundant guanine nucleotide exchange factor (eIF-2B, aka GEF), thereby preventing the recycling of eIF-2 (8, 9).

In previous work we asked whether or not eIF-2α phosphorylation is the primary cause or a sufficient condition for translation inhibition. The putative phosphorylation sites on eIF-2α, Ser48 and Ser51, each were altered by site-directed mutagenesis (10, 11) to alanine residues which are expected to prevent phosphorylation. The Ser48Ala variant (2α-AS) was phosphorylated in vitro by HRI and DAI, whereas the Ser51Ala variant (2α-SA) was not, suggesting that the site of phosphorylation for these kinases is Ser51 (10). The 2a variants as well as the Ser48Asp (2α-DS) and Ser51Asp (2α-SD) variants (designed to mimic phosphorylation at either site) were tested for possible effects on translation in intact cells. Wild-type and mutated cDNAs were individually subcloned in a mammalian expression vector and cotransfected into COS-1 cells with a dihydrofolate reductase (DHFR) expression vector. The expression level of DHFR was monitored by densitometry of the electroblot of the DHFR protein. The data indicate that the two variants Ser48Ala and Ser51Asp do not significantly affect the expression level of DHFR (2). This study was supported in part by National Institutes of Health Grant GM22135 from the United States Public Health Service. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.

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1 The abbreviations used are: eIF, eukaryotic initiation factor; DHFR, dihydrofolate reductase; DME, Dulbecco’s minimal essential medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodeyl sulfate; NEPHGE, nonequilibrium pH gel electrophoresis; IEF, isoelectric focusing; VS-IEF, vertical slab isoelectric focusing; PAGE, polyacrylamide gel electrophoresis; Met-tRNA, initiator methionyl-tRNA; ds, double-stranded; BSA, bovine serum albumin; FPLC, fast protein liquid chromatography; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; HRI, hemin-regulated inhibitor; DAI, double-stranded RNA-activated inhibitor.

2 All of the eIF-2α variants described in this study involve changes at the Ser codons at positions 48 and 51. A simplified nomenclature is used that cites sequentially the residue at positions 48 and 51. Thus, the wild-type protein is named 2α-SS, the single variant Ser48Ala is named 2α-AS, etc.
expression vector, pD61 (11). DHFR translation from mRNA expressed from pD61 is inefficient due to activation of DAI kinase in the transfected cells (7). Cotransfection with the 2a-SA variant stimulates DHFR synthesis, apparently by preventing phosphorylation or otherwise interfering with the effects of an activated DAI kinase. In contrast, cotransfection with the 2a-SD, but not the 2a-DS, variant completely inhibits DHFR translation, indicating that phosphorylation at Ser51 but not Ser48 is sufficient for translational repression in these cells. Unexpectedly, the 2a-AS variant also stimulates DHFR synthesis, even though the 2a-AS protein can be phosphorylated (10, 11). This surprising result suggests that the mechanism of action of the overproduced variant proteins is more complicated than initially envisaged. It also has been argued that phosphorylation at both Ser51 and Ser48 may be required for translation inhibition (12).

In order to understand better the mechanism of variant eIF-2a action in the transiently transfected COS-1 cells, we asked whether or not the over-expressed free eIF-2a proteins serve as HRI and DAI substrates or directly inhibit these kinases in vivo, whether or not they exchange into the endogenous COS cell eIF-2 heterotrimeric complexes, and to what extent the eIF-2a subunit is phosphorylated, either free or in the eIF-2 complexes. In addition, we have examined the expression of double variants of eIF-2a to ascertain the physiological importance of both Ser51 and Ser48, thereby defining the molecular basis for the action of this initiation factor in translational regulation.

MATERIALS AND METHODS

Construction of Plasmids and Site-directed Mutagenesis—All recombinant DNA techniques were performed by standard methods (13) except as noted. The DHFR expression plasmid pD61 and the eIF-2a expression plasmid pMT2VA contain the DHFR cDNA, but lack adenovirus VA-1 and VA-II genes as described (11). Plasmids pE2-2aVA, p48A-VA, p51A-VA, and p51D-VA, which express the 2a-SA, 2a-AS, 2a-SA, and 2a-SD variants of eIF-2a in pMT2VA, respectively, were previously described (11).

Oligonucleotide-directed mutagenesis was performed to generate Ser51Ala-Ser51Ala (2a-AS) and Ser51Ala-Ser48Asp (2a-AD) double variants of eIF-2a by the gapped duplex DNA method (14) using PCR or plasmid vectors. Human eIF-2a cDNA encoding 2a-AS (11) in pUC18 was used as the target DNA fragment. A 23-mer oligonucleotide (5′-CG-CCT-TCT-G(G/T)C-CAA-TTC-AGC-AAG-3′) was synthesized and used to mutate TTAT-GCC into TTG-GCC or TTG-GAG resulting in the substitution of an Ala or Asp for Ser51, respectively (the nucleotides corresponding to codon 51 are underlined).

The 2a-AS variant was designed to create a Bal31 restriction site. Mutated cDNAs were screened and selected by sequencing with an oligonucleotide primer (5′-CT-GCT-AAT-TGC-GAT-GAG-3′) by the dideoxyoligonucleotide termination method (15). The EcoRI-HincII fragments of 2a-AS and 2a-AD cDNA from the pm vector were blunt ended with the Klenow fragment of Escherichia coli DNA polymerase, ligated with EcoRI linkers, and digested with EcoRI. The fragments were purified by agarose gel electrophoresis and subcloned into the unique EcoRI site of pmT2VA to obtain pMT2-2a-AS or pMT2-2a-AD. The correct orientation and structure of both clones were confirmed by restriction enzyme digestion and gel electrophoresis.

Cell Culture and Transfection—COS-1 cells grown on 100-mm plates were transfected by the DEAE-dextran method (11). Briefly, approximately 70% confluent plates of cells were fed 4 ml of Dulbecco’s minimal essential medium (DMEM) containing 1 mg of DEAE-dextran (Pharmacia LKB Biotechnology Inc.) and 8 μg of eIF-2a expression plasmid and/or 8 μg of pD61/plate for 6–12 h. After washing plates with DMEM or phosphate-buffered saline, the monolayer cultures were treated with 10% dimethyl sulfoxide for 2 min followed by 0.1 mM chloroquin in DMEM with 10% fetal bovine serum for 2 h. Plates were then incubated with fresh DMEM plus serum. To monitor DHFR synthesis and exchange of eIF-2a into eIF-2 complex (see below), cells were pulse-labeled with [35S]methionine (100 μCi/ml; >1000 Ci/mmol, Amersham Corp.) for either 30 min or 8 h beginning at 40-h post-transfection. After 48- or 72-h post-transfection, cells were harvested for IEF-PAGE gel analysis of total cell proteins by direct addition of an ampholine solution (9.8 μm urea, 2% Nonidet P-40, 2% pH 3–10 Ampholines, 25 mM NaF, and 1% 2-mercaptoethanol) as described (16). For analyses under non-denaturing conditions, cells were washed with cold phosphate-buffered saline, pelleted, and stored immediately at −70 °C until further use.

Separation of the eIF-2 Complex from Free eIF-2a Subunits by Gel Filtration—Gel filtration column chromatography was performed using a Pharmacia FPLC system to separate the COS cell eIF-2 trimeric complex from free overproduced human eIF-2a subunit. Cell pellets from one 100-mm plate (~2 × 107 cells) were suspended in 200 μl of non-denaturing lysis buffer (20 mM HEPES, pH 7.5, 50 mM KCl, 1 mM EDTA, 25 mM NaF, 7 mM 2-mercaptoethanol, 5% glycerol, 0.5% Nonidet P-40), and subsequently lysed by 25 strokes in a Dounce homogenizer. Bovine serum albumin (BSA) (30 μg) was added as carrier protein. The cell lysate was centrifuged at 10,000 × g for 10 min to obtain a post-mitochondrial supernatant. The supernatant was transferred into a siliconized Eppendorf tube with 30 μg of lysozyme added as carrier protein, adjusted to 500 mM KCl, and loaded on a Pharmacia HR 10/30 Superose-12 FPLC column. The column was previously washed with buffer H(500) (20 mM HEPES, pH 7.5, 50 mM KCl, 50 mM NaF, 50% glycerol, 100 μM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 10% glycerol) and eluted at 0.3 ml/min. Fractions (0.5 ml) were collected in siliconized Eppendorf tubes supplemented with 100 μg of lysozyme, quickly frozen, and stored at −70 °C. The fractions containing eIF-2 complex (fractions 22–24) or free eIF-2a subunit (fractions 25–26) were determined in separate experiments by fractionation of the COS-1 cell lysate together with HeLa cell lysate (2 × 107 cells) and immunoblotting with affinity-purified antibodies to eIF-2a and eIF-2γ (17). Such an elution profile of eIF-2 and free eIF-2a subunits from the Superose-12 column is illustrated in Fig. 1. For the routine preparation of eIF-2 complexes and free eIF-2a subunits, transfected cells from 10 plates (~2 × 108 cells total) were used, and no HeLa cell lysate was added.

Purification of Overexpressed eIF-2a—The initial steps of purification of eIF-2a subunits were identical to those described above. Peak fractions from the Superose-12 column of free eIF-2a subunits were pooled and supplemented with the final concentration of 100 mM KCl, and the salt concentration was reduced to 100 mM by dialution with H(00) buffer (identical to H(500) but lacking KCl). The diluted sample was then immediately applied to a Pharmacia HR 5/5 MonoQ FPLC column previously equilibrated with H(100) buffer. The column was washed with H(100) buffer and then developed with a linear gradient of 100–500 mM KCl in the same buffer. eIF-2a eluted at 260 mM KCl as determined by immunoblotting with affinity-purified anti-eIF-2a antibody (17). The eIF-2a subunit was >90% pure as determined by SDS-PAGE and staining with Coomassie Blue (results not shown). Its protein concentration was determined by comparison of staining band intensities with those generated by known amounts of purified eIF-2 (18) and by immunoblotting.

In Vitro Phosphorylation of Overexpressed eIF-2a—Purified free eIF-2a subunits from the MonoQ column were phosphorylated by HRI or DAI kinase in the presence or absence of purified HeLa eIF-2 (18). HRI was highly purified through the last step of the procedure described by Jackson and Hunt followed by passage through a MonoS column to remove casein kinase II activity (19); DAI was highly purified through the MonoS column to remove casein kinase II activity (19)); DAI was highly purified through the MonoS column to remove casein kinase II activity (19); DAI was highly purified through the MonoS column to remove casein kinase II activity (19)).

The same fractions of eIF-2a were also phosphorylated by DAI. The reaction mixtures (25 μl) contained 25 mM HEPES, pH 7.4, 100 mM KCl, 1 mM MgCl2, 0.3 mg/ml BSA, 50 μM [γ-32P]ATP (>1 × 106 cpm), and purified eIF-2α and/or HeLa eIF-2 as indicated in Fig. 2. After 15 min at 30 °C, the reaction was quenched by the addition of 4 × SDS buffer and the extent of phosphorylation was monitored by 10% SDS-PAGE (21) and autoradiography.

The same fractions of eIF-2a were also phosphorylated by DAI. The reaction mixtures (30 μl) contained 20 mM HEPES, pH 7.4, 75 mM KCl, 5 mM MgCl2, 0.3 mg/ml BSA, 80 ng/ml poly(I).poly(C), 50 μM [γ-32P]ATP and purified eIF-2α and/or HeLa eIF-2 as indicated in Fig. 2. After 15 min at 30 °C, the reaction was quenched by the addition of 4 × SDS buffer and the extent of phosphorylation was monitored by 10% SDS-PAGE (21) and autoradiography.
indicated in the figures. The identification of spots corresponding to eIF-2α subunits was by immunoblotting as described below.

One-dimensional VS-IEF gel electrophoresis was carried out as described (23, 24) with modifications. The gel (150 x 170 x 0.8 mm) contains 9.8 M urea, 4% (w/v) Servalytes pH 5–6/pH 3–10, mixed in the ratio 3:1, 2% (w/v) CHAPS (Boehringer-Mannheim), and 5% (w/v) polyacrylamide, and pre-run at 200 V for 45 min, then at 300 V for 45 min, before sample application. Electrode solutions were 50 mM histidine at the cathode and 10 mM glutamic acid at the anode. Samples were prepared with the ampholyse containing 9.8 M urea, 2% (w/v) Servalytes (3:1 pH 5-6/pH 3-10), 2% (w/v) CHAPS, and 1% 2-mercaptoethanol, applied in each well, and overlayed with 75% ampholyse. After isoelectric focussing at 1.5 mA for 20 h, the proteins were electrotransferred to Immobilon (Millipore) as described (16). The blots were incubated with affinity-purified rabbit anti-eIF-2α, were electrotransferred to Immobilon (Millipore) as described (16).

Samples were prepared with the ampholyse containing 9.8 M urea, 2% (w/v) Servalytes (3:1 pH 5-6/pH 3-10), 2% (w/v) CHAPS, and 1% 2-mercaptoethanol, applied in each well, and overlayed with 75% ampholyse. After isoelectric focussing at 1.5 mA for 20 h, the proteins were electrotransferred to Immobilon (Millipore) as described (16). The blots were incubated with affinity-purified rabbit anti-eIF-2α, a goat anti-rabbit IgG antibody (Cappel) conjugated with alkaline phosphatase, and visualized as described (25) with minor alterations. 125I-Labeled goat anti-rabbit IgG also was used to detect eIF-2α by autoradiography. Affinity-purified antibodies were prepared as described (17), except that aminophenylthioether cellulose paper (Schleicher & Schuell) was used for preparation of anti-eIF-2α and -2γ chips.

RESULTS

Interaction of Free eIF-2α Subunits with Kinases—Over-expression of variant forms of eIF-2α, where Ala is substituted for Serα (2α-AS) or Ser2α (2α-SA), stimulates translation of plasmid-derived mRNAs after transfection of COS-1 monkey cells (11). A possible mechanism whereby these alanine variant forms stimulate protein synthesis is through direct inhibition of DAI kinase. Peptide analogues of phosphorylation sequences where Ser is replaced by Ala often function as specific inhibitors of their kinases (26). Since eIF-2α is over-expressed in transiently transfected COS-1 cells and accumulates to levels 10–20-fold greater than that of the endogenous eIF-2α trimeric complex (11), the excess free subunit of the factor may function as an inhibitor of DAI or other eIF-2α kinases. In order to test directly this possibility, free 2α-SS, 2α-AS, and 2α-SA subunits were purified from transiently transfected cells. Cell proteins were fractionated as described under "Materials and Methods" by FPLC chromatography on a Superose-12 column to separate endogenous COS cell eIF-2 from the free human eIF-2α subunit based on their differences in size. As shown in Fig. 1, the eIF-2 complex and free eIF-2α subunit were identified in different fractions by immunoblotting with affinity-purified antibodies against eIF-2α and eIF-2γ.

The wild-type and variant eIF-2α subunits were used to determine whether the free subunit is a substrate for eIF-2α kinases and whether it directly inhibits phosphorylation of the eIF-2 complex. We performed in vitro phosphorylation reactions as described under "Materials and Methods," by addition of HRI to reaction mixtures containing the partially purified free eIF-2α subunit prepared by Superose-12 chromatography. Reaction conditions were optimized for HeLa eIF-2, but an unknown potent HRI inhibitor in the eIF-2α fractions inhibited both the autophosphorylation of HRI and the phosphorylation of eIF-2. Comparable Superose-12 fractions from control lysates of COS-1 cells transfected with a vector (pD61) lacking an eIF-Pa insert also inhibited the kinase (data not shown), suggesting that the eIF-2α subunit is not responsible for the inhibition. For this reason, the free eIF-2α subunit from Superose-12 was further purified by using a MonoQ column as described under "Materials and Methods." This procedure removes the HRI inhibitor and generates eIF-2α preparations that are >90% pure (results not shown). Fig. 2A demonstrates that both the wild-type 2α-SS (lanes 8 and 9) and the variant 2α-AS (lanes 12 and 13) forms are phosphorylated weakly, and thus are poor substrates for HRI. The 2α-SA variant form (lanes 16 and 17) is not phosphorylated at detectable levels, consistent with prior results (10).

Next, we tested whether the free eIF-2α forms might inhibit HRI phosphorylation of the eIF-2 complex. The effects of the wild-type and variant forms were monitored in reaction mixtures containing HeLa eIF-2 substrate, a limiting amount of HRI, and varying amounts of the free eIF-2α subunits. The relative amounts of HeLa eIF-2 and the purified eIF-2α subunits were carefully determined by comparison of Coomasie Blue-stained band intensities and immunoblot intensities. As shown in Fig. 2A (lanes 10, 11, 14, 15, 18, and 19), none of the free 2α-subunits inhibits the phosphorylation of HeLa eIF-2 or the autophosphorylation of HRI, even at up to 8-fold molar excess. The intensity of radioactivity in the eIF-2α band approximates the sum of the intensities of eIF-2 alone (lane 3) plus the free subunit alone.

In another set of experiments, the same fractions of free eIF-2α subunits were tested as substrates or inhibitors of DAI activated with poly(I).poly(C) (Fig. 2B). In this set of phosphorylation reactions, the HeLa eIF-2 purified on MonoQ and MonoS columns was contaminated with a potent inhibitor of DAI activation. When HeLa eIF-2 was added to DAI prior to its activation with dsRNA, the autokinase and eIF-2α kinase activities of DAI were completely inhibited (lane 18). However, when dsRNA is added before the eIF-2 substrate, no inhibition was observed, suggesting that the inhibitor prevents the activation of DAI by interfering with the
Stimulation of Protein Synthesis by eIF-2α Variants

**Overexpressed eIF-2α Subunits Exchange into Endogenous eIF-2 Complexes**—An alternative mechanism to explain the stimulation of protein synthesis by 2α-AS and 2α-SA is the incorporation of the variant forms into the cell’s eIF-2 complex by de novo synthesis and assembly and by exchange into preexisting complexes. Since transiently transfected cells are no longer growing exponentially when overproduction of eIF-2α occurs, the contribution of de novo assembly may be relatively minor compared to exchange into endogenous eIF-2 complexes. A strategy to determine if exchange occurs is to pulse-label transfected cells with \(^{35}S\)methionine and then measure the specific radioactivities of the α- and γ-subunits in the endogenous eIF-2 complex previously separated from free eIF-2α subunits. Radioactivity in the γ-subunit is a measure of de novo synthesis and assembly into eIF-2 during the pulse, and a comparable amount of radioactivity is expected for the other subunits if exchange does not occur. The specific activity of the free, overexpressed eIF-2α subunit will be much higher since its rate of synthesis is greater and there is little or no endogenous free subunit to dilute its specific radioactivity. Therefore, if exchange occurs, the specific activity of the α-subunit in the eIF-2 complex should be higher than that of the other eIF-2 subunits. To determine whether or not exchange occurs, pulse-labeled COS-1 cells transiently expressing eIF-2α were lysed and the eIF-2 complex was partially purified by FPLC Superose-12 chromatography as described previously for the preparation of the free eIF-2α subunit. Three fractions containing the trimeric eIF-2 complex were pooled and analyzed by NEPHGE-SDS-PAGE which separates the α- and γ-subunits from other COS-1 proteins (Fig. 3). In control cells transfected with pD61 which lacks eIF-2α cDNA, the intensities of the spots for eIF-2γ and eIF-2α are both very weak, as expected. However, in analyses of fractions containing the eIF-2 complex from 2α-SS, 2α-AS, and 2α-SA transfected cells, the specific radioactivity of the α-subunit is 10-20-fold higher than that of the γ-subunit as determined by visual inspection. We conclude that very extensive exchange of the eIF-2α subunit occurs between the complex and free forms. Thus, in the case where a variant form of eIF-2α is overexpressed, essentially all of the endogenous eIF-2 complex in the transfected cells would contain the variant form of the subunit. With the 2α-SA mutant, it seems highly likely that such eIF-2 complexes cannot be phosphorylated by HRI or DAI, since the free form is not phosphorylated, and that protein synthesis would continue uninhibited even in the presence of activated eIF-2α kinases. However, with the 2α-AS variant, it is possible that such eIF-2 complexes are readily phosphorylated. If so, why does overexpression of the 2α-AS also reverse the inhibition of protein synthesis caused by the action of DAI?

**Analysis of Double Variant Forms of eIF-2α in COS-1 Cells**—In order to shed more light on the effects of mutating the Ser codons at positions 48 and 51 of eIF-2α, two double mutations of the cDNA were constructed. The eIF-2α cDNA was altered at both codons 48 and 51 by site-directed mutagenesis as described under “Materials and Methods,” so that it expresses either an Ser⁴⁸Ala-Ser⁵¹Ala form (2α-AA) or an Ser⁴⁸Ala-Ser⁵¹Asp form (2α-AD). These double variants serve to evaluate whether or not eIF-2α is phosphorylated at both Ser⁴⁸ and Ser⁵¹, as proposed recently (12), and to assess their ability to stimulate or inhibit protein synthesis.

The effect of expression of the various variant eIF-2α forms was determined by cotransfection with a reporter gene which expresses DHFR (pD61). Expression of DHFR from mRNA transcribed from pD61 is inefficient due to DAI kinase activation in transfected cells (7). pD61 was cotransfected with

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**Fig. 2. In vitro phosphorylation of free eIF-2α subunits with eIF-2 kinases.** Purified free 2α-SS, 2α-AS, and 2α-SA subunits (>90% pure; prepared by elution from MonoQ columns) were phosphorylated by HRI (panel A) or DAI (panel B) kinase with [γ-³²P]ATP in the presence or absence of purified HeLa eIF-2. The extent of phosphorylation was monitored by 10% SDS-PAGE and autoradiography. Panel A, 1 pmol of purified HeLa eIF-2 and 2α-SS and 3α-SS molar excess of each type of free eIF-2α subunit were added to reaction mixtures (25 μl) as indicated. In the case of preparations from pMT2-transfected cells (lanes 4–7), no free eIF-2α was detected in the MonoQ fractions; 3x and 8x refer to the same volumes used in the 2α-SS transfected preparations. Panel B, 1 pmol of HeLa eIF-2 and 1–8-fold molar excess of each eIF-2α subunit were added to reaction mixtures (30 μl) containing DAI and dsRNA (poly(I)·poly(C), 80 ng/ml) as indicated. In lane 18, HeLa eIF-2 was added to the mixture prior to the addition of DAI. Free eIF-2α preparations contain an unknown containing kinase (lanes 1, 3, 11, 13, 15, and 17). Molecular weight markers (in kDa) are shown on the left in panel B. Bands corresponding to eIF-2α and the autoprophosphorylated HRI and DAI kinases are labeled on the right.

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Table 1: Summary of Results

| Variant Form | Synthesis and Assembly | Exchange into Endogenous Complex |
|--------------|------------------------|----------------------------------|
| 2α-SS        | 2α-AS                  | 2α-SA                            |
|              |                        |                                  |
|              |                        |                                  |
|              |                        |                                  |

| Kinase | eIF-2α Subunit | eIF-2γ Subunit | eIF-2δ Subunit |
|--------|----------------|----------------|----------------|
| HRI    | 2α-SS          | 2α-AS          | 2α-SA          |
| DAI    | 2α-SS          | 2α-AS          | 2α-SA          |

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**Binding of dsRNA to DAI.** In contrast to the results with HRI, 2α-SS and 2α-AS are good substrates for DAI (lanes 7 and 8); however, as seen with HRI, 2α-SA is not phosphorylated (lane 9). However, when an 8-fold greater amount was tested, appreciable phosphorylation was detected (lane 16); this extent of phosphorylation very likely is due to COS cell eIF-2α that has exchanged out of the cell’s endogenous eIF-2 complex (see below). The phosphorylation of the 2α-SS and 2α-AS subunits almost reached the level of phosphorylation of the α-subunit in the HeLa eIF-2 complex (compare lanes 7 and 8 to lane 6). None of the free eIF-2α subunits (up to 8 x molar excess) acts as an inhibitor of DAI in these reactions. On the basis of the in vitro phosphorylation results, we conclude that stimulation of translation of plasmid-derived mRNA by the 2α-AS and 2α-SA variant forms does not result from the direct inhibition of eIF-2α kinases by the high level of overproduced free subunits.
cell extracts were fractionated by Superose-12 chromatography. The 290 cells (2 μg MT2VA- carrying the cDNAs of 2a-SS, 2a-AS, and 2a-SA, as described under “Materials and Methods.” At 40-h post-transfection, cells (-2 × 10⁶) were pulse-labeled with [35S]methionine for 8 h, and cell extracts were fractionated by Superose-12 chromatography. The fractions containing eIF-2 complex separated from free eIF-2a subunits were combined and analyzed by NERFGE and autoradiography. Migration was from the acidic region (right) toward the basic region (left). Only a portion of the autoradiogram is shown. Arrows point to the γ-subunit (upper left of each panel) and α-subunit (lower right) as labeled in the D61 panel.

The wild-type and different Ser variants of eIF-2a contained in the expression vector pMT2VA-. Cells were pulse-labeled with [35S]methionine and cell extracts were analyzed by SDS-PAGE. For wild-type and most variants, eIF-2a synthesis is readily detected by analysis of the total protein (Fig. 4, top left panel), eIF-2a synthesis was quantitated by immunoprecipitation and SDS-PAGE (top right panel). As shown previously (11, 27), similar levels of eIF-2a synthesis were detected from the wild-type 2a-SS (lane 9) and the 2a-AS (lane 10) and 2a-SA (lane 11) variants; the double variant 2a-AA (lane 12) is equally well synthesized. Northern blot analysis using a DHFR-specific probe which hybridizes to the 3′ end of the eIF-2a mRNA shows equal amounts of mRNA in each of these transfected cell populations (Fig. 4, bottom panel), demonstrating that these variants are translated with equal efficiency. In contrast, expression of the 2a-SD (lane 14) and 2a-AD (lane 15) is significantly reduced. For these immunoprecipitation reactions (lanes 13–15), 10 times the amount of trichloroacetic acid-precipitable counts were immunoprecipitated in order to detect eIF-2a synthesis. Quantitation of the band intensities after subtraction of the endogenous level of eIF-2a synthesis (lane 13) demonstrates that 2a-SD synthesis is reduced 200-fold compared to wild-type eIF-2a, consistent with results reported previously (11), and 2a-AD synthesis is reduced 35-fold. Quantitation of eIF-2a mRNA in the transfected cells shows a 4-fold reduction for 2a-SD and a 2.5-fold reduction for 2a-AD compared to 2a-SS. This reduction in mRNA level was reproducible in separate experiments and may result from a secondary effect of inhibition of protein synthesis in cells expressing 2a-SD or 2a-AD. Corrected for the cellular levels of eIF-2a mRNA, the translation of 2a-SD late in transfection is reduced 50-fold compared to wild-type. Significantly, translation of 2a-AD is reduced only 14-fold compared to wild-type. Thus, mutation of the Ser to Ala codon for residue 48 decreases approximately 3.5-fold the inhibitory effect of the Asp residue on the translation rate. The effect of wild-type and variant eIF-2a expression on DHFR translation was examined by analysis of DHFR synthesis detected in the cell extracts. DHFR is detected as a 20-kDa protein by SDS-PAGE of lysates from pD61-transfected cells. Cotransfection with either variant 2a-AS or 2a-SA (lanes 3 and 4) stimulates DHFR synthesis compared to the wild-type 2a-SS (lane 2), as previously observed (11). Cotransfection with the 2a-AA variant also stimulates DHFR synthesis to a similar degree as that observed with either of the single Ser to Ala variants (lane 5). Since similar levels of DHFR mRNA are present in all of these cotransfected cells (Fig. 4, bottom panel), the differences in DHFR synthesis result from differences in translational efficiency. These results show that prevention of phosphorylation at both residues 48 and 51 does not cause additional stimulation of protein synthesis over that observed with either Ala replacements alone. It was not possible to detect DHFR synthesis in cells cotransfected with either the 2a-AD (lane 7) or the 2a-AD (lane 6) variants, suggesting that phosphorylation at Ser
(mimicked by the Asp residue) is sufficient to inhibit protein synthesis.

The phosphorylation states of the overexpressed eIF-2α subunit forms were evaluated by immunoblotting to assess the effects of the various substitutions at Ser. The free eIF-2α subunits were partially purified by FPLC Superose-12 chromatography, then subjected to VS-IEF-PAGE and immunoblotting. Overexpression of either the wild-type or Ala variant cDNAs results in the accumulation of large amounts of free 2α-subunits compared to that in pD61-transfected cells lacking eIF-2α cDNA (Fig. 5). The ratio of phosphorylated (right band) to non-phosphorylated (left band) protein is comparable for the wild-type and all Ala variant forms, and lies in the range of 0.1–0.15. The presence of low amounts of phosphorylated eIF-2α in the transfection with the 2α-AA cDNA requires explanation, since phosphorylation of this protein is prevented at both residues 48 and 51. It is unlikely that non-transfected cells contribute significant amounts of free phosphorylated 2α-subunit since very little free eIF-2α is detected in cells that receive pD61 alone. It is more likely that the phosphorylated protein results from wild-type eIF-2α that exchanges out of the endogenous COS cell eIF-2 complex in cells that overexpress the 2α-subunit. Evidence for such exchange has been presented in Fig. 3, and also is seen in Fig. 5, lanes 6 and 7, where moderately intense non-phosphorylated eIF-2α bands are seen that must be due to exchanged endogenous COS cell eIF-2α subunits. A third, as yet unidentified, phosphorylation site also might contribute to the overall phosphorylation pattern of the Ala mutants. Since the phosphorylation pattern is comparable for cells transfected with both 2α-SA and 2α-AA, it is apparent that the 2α-SA subunit is not appreciably phosphorylated on Ser48.

Analysis of the 2α-SD and 2α-AD forms shows some non-phosphorylated eIF-2α and a new band with an isoelectric point slightly less acidic than phosphorylated eIF-2α (Fig. 5), consistent with an Asp replacement of Ser. The intensity of the 2α-AD band is much greater than the corresponding band (lane 15) in Fig. 4. This is due to the fact that Fig. 5 measures the total accumulation of free eIF-2α over about 2 days, whereas Fig. 4 measures the rate of eIF-2α synthesis late in transfection when the effects of accumulated variants are most apparent. The lower abundant protein corresponding to non-phosphorylated eIF-2α must be due to COS cell eIF-2α subunits, by an exchange mechanism and possibly from non-transfected cells. No band more acidic than 2α-AD or 2α-SD is detected for either variant form. This result shows that neither 2α-SD nor 2α-AD is phosphorylated to a significant extent. The importance of this observation in terms of the number of phosphorylations required for transpositional repression is discussed below.

**Analysis of the in Vivo Phosphorylation State of eIF-2α**

Since the free eIF-2α variant forms do not inhibit HRI or DAI activities in vitro, and since they freely exchange into endogenous COS cell eIF-2 complexes, it is highly likely that the Ala and Asp variants affect translation by converting the endogenous eIF-2 complex into a variant form. It is therefore important to evaluate the extent of phosphorylation of the 2α-subunit in the eIF-2 complex. This is not a trivial matter because only up to 30% of the cells are actually transfected, which results in about 70% of the eIF-2 in a cell preparation coming from unaltered (wild-type) eIF-2. However, since the specific activity of the 2α-subunit is high because of overexpression, analysis of radioactivity rather than mass will reflect the status of eIF-2α in essentially only the transfected cell population. Cells were cotransfected with eIF-2α wild-type or mutated cDNAs and with pD61 (to activate DAI in order to elicit phosphorylation) and labeled with [35S]methionine for 8 h at 40 h post-transfection. Subsequently, the eIF-2 complexes were separated from free, overproduced 2α-subunits as described above. The fractions containing the eIF-2 complexes were then subjected to two-dimensional IEF/SDS-PAGE to separate and identify phosphorylated and non-phosphorylated 2α-subunits. As shown in Fig. 6, the more intense 2α-subunit for 2α-SS, 2α-AS, and 2α-SA confirms the overproduction and exchange of these proteins into the complex. Moderate phosphorylation of 2α-SS and 2α-AS is observed. This suggests that replacement of Ser for Ala at position 48 does not significantly influence the extent of phosphorylation of Ser at position 51. In contrast, no distinct spot of enhanced intensity corresponding to the phosphorylated form of 2α-SA or 2α-AA is detected, as expected.

In the case of 2α-SD and 2α-AD, a new spot, slightly less acidic than phosphorylated eIF-2α, is seen which corresponds to the variant protein. It is apparent that the variant eIF-2α

![Fig. 5. Analysis of the free eIF-2α fractions by VS-IEF-PAGE](#)

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![Fig. 6. IEF/SDS-PAGE analysis of in vivo phosphorylation of wild-type and variant eIF-2α subunits in eIF-2 trimeric complexes](#)
subunits have exchanged into endogenous eIF-2. Since the variant form of the eIF-2 complex mimics the structure of the phosphorylated factor, it is expected to inhibit all of the eIF-2B in the cell since eIF-2B is thought to be present at much lower cellular levels than eIF-2 (28). This is indeed observed in Fig. 4, where strong repression of DHFR synthesis occurs with these variants.

**DISCUSSION**

The inhibition of protein synthesis by the phosphorylation of eIF-2 is the best characterized translational control mechanism operating at the level of covalent modification of translational components. Phosphorylation of the α-subunit correlates with repression in numerous physiological conditions; a convincing change in factor activity has been demonstrated in vitro, and two highly specific protein kinases have been identified and their control has been characterized (reviewed in Ref. 3). Evidence that eIF-2α phosphorylation is the actual cause of translation inhibition in vivo has been more difficult to obtain. The fact that the activation of two different protein kinases with high specificity for eIF-2α both lead to inhibition of initiation is strongly suggestive, but there is always uncertainty that other as yet unidentified substrates may be critically important for the repression. To resolve this ambiguity, variant forms of eIF-2α were expressed in transiently or long term transfected cells in order to alter the extent of translation inhibition upon activation of the eIF-2α kinase, DAI (11, 27). The findings that expression of the 2α-SD variant inhibits total protein synthesis whereas expression of the 2α-SA variant mitigates the inhibition provide the best evidence that eIF-2α phosphorylation truly causes translational repression in vivo. However, an unexplained observation is that whereas the 2α-DS variant causes no inhibition (suggesting that phosphorylation at Ser45 does not lead to inhibition), the 2α-AS variant is as active in decreasing translation inhibition as is the 2α-SA variant in cells where DAI is activated (11, 27). Therefore, the work described here was undertaken to increase our understanding of how the overexpressed eIF-2α variant proteins actually function to alter translation rates.

An important first step was to determine whether or not the free eIF-2α subunit is itself active in these cells. We obtained highly purified, overexpressed eIF-2α subunits from transiently transfected COS-1 cells by employing rapid FPLC chromatographic methods. The 2α-SS wild-type and 2α-AS variant subunits serve as substrates for HRI and DAI, although in the case of HRI they are considerably less active than the purified HeLa eIF-2 complex. That these proteins are phosphorylated at all is of interest, since the 2α-subunit obtained by dissociation of eIF-2α with denaturing buffer is not a substrate. Apparently, the conformation of the protein is important for its recognition by the protein kinases, suggesting that the accumulated, overexpressed subunits possess a conformation comparable to that in the eIF-2 complex. Although 2α-SS and 2α-AS are poor substrates for HRI and 2α-SA is not phosphorylated appreciably, none of these proteins inhibits the phosphorylation of the eIF-2 trimeric complex, even when tested in 8:1 molar excess. It therefore is very likely that the free mutant eIF-2α subunit, which accumulates up to 10–20 mol/mol of endogenous COS cell eIF-2, does not inhibit activated DAI in transfected cells (11, 27). Our biochemical results are consistent with the finding that DAI assayed in vitro in transfected cell lysates is active on exogenous eIF-2 substrate (27). In effect, no regulatory role for the free eIF-2α subunits was demonstrated.

The possibility remained that the active form of the variant proteins involves the endogenous eIF-2 trimeric complexes, generated by an exchange mechanism that replaces the endogenous 2α-subunit with the variant form. Our results indicate that an efficient exchange process occurs in vivo for 2α-SS and all of the variants. The precise rate of exchange is not known, but greater than 90% exchange must occur within the 8 h (and sometimes 5 h) labeling periods employed in the experiments. An active exchange implies that eIF-2 may be in equilibrium with all three of its subunits. However, the failure to readily detect free eIF-2α subunits in cells transfected only with pD61 and not with the eIF-2α cDNA expression vector suggests that significant levels of free subunits do not occur in normal cells. Exchange of eIF-2α into the eIF-2 complex was unexpected because biochemical characterization of eIF-2 indicates a very strong complex that resists dissociation into subunits. The exchange phenomenon is consistent with, but does not prove, a mechanism of action of eIF-2 involving a transiently free form for the eIF-2α subunit, as suggested by Gupta and co-workers (29).

A likely mechanism of action of the variant forms is to alter the extent of phosphorylation of the eIF-2 complex. eIF-2 containing the 2α-SA variant is expected to resist phosphorylation and thereby evade the inhibition potentially caused by DAI. To explain the similar effects of the 2α-AS variant, we hypothesized that although the 2α-AS protein can be phosphorylated on Ser51, the relative activities of the DAI and phosphoprotein phosphatases are such that the equilibrium with the variant would be shifted to less phosphorylation, thereby resulting in an eIF-2 that does not repress translation. However, examination of the extent of 2α-AS subunit phosphorylation in the eIF-2 trimeric complex by IEF/SDS-PAGE (Fig. 6) indicates an amount of phosphorylation comparable to that of the wild-type subunit, which does not reverse the effects of DAI so effectively. Two features of this experiment complicate the interpretation of the results. First, it is necessary to separate the free 2α-subunits from the eIF-2 complex, a step involving non-denaturing conditions during which the extent of phosphorylation can change. The lysate preparation and Superose-12 chromatography were performed in buffers designed to inhibit both kinase and phosphatase action, but it is difficult to rule out some changes in the extent of phosphorylation of the partially purified eIF-2 complex during purification and analysis. Second, only 20–30% of the cell population is transfected, resulting in only a small fraction of eIF-2 complexes containing the variant form. However, since transfected cells contain high specific radioactivity in eIF-2α, and thus contribute the major part of the radioactivity in the eIF-2α spots, the radioactive patterns reflect phosphorylation primarily in transfected cells. The results strongly suggest that the 2α-AS mutant stimulates translation by a mechanism other than altering the extent of phosphorylation.

A possibility is that phosphorylation of eIF-2α occurs on two sites, namely Ser51 and Ser45, both essential for inhibition as argued recently by Kramer (12). If this were true, then both the 2α-AS and 2α-SA variants would resist the double phosphorylation and thereby stimulate protein synthesis, as is observed (11). However, the data reported here do not support the view that simultaneous phosphorylation on both Ser45 and Ser51 occurs. Analysis of IEF-PAGE of the free 2α-subunit from cells transfected with wild-type 2α-SS shows extensive monophosphorylation but no discernable band corresponding to a second phosphate (Fig. 5). A comparable amount of monophosphorylated eIF-2α is seen when the variant 2α-AA is expressed, even though the variant subunit cannot be phosphorylated at positions 48 and 51. The phosphorylated 2α-AA subunit seen with 2α-AA is very likely due to the wild-type COS cell 2α-subunit that has exchanged out of

**Stimulation of Protein Synthesis by eIF-2α Variants**
the endogenous eIF-2 complex; a small contribution by phosphorylation at a third site cannot be ruled out, however. The single Ala variant, 2a-SA, shows a phosphorylation pattern essentially identical to the 2a-AA variant, indicating that a negligible amount of phosphorylation at Ser48 occurs on this protein. Furthermore, the variants 2a-SA, 2a-AS, and 2a-AA prevent the inhibitory action of DAI equally effectively. Even the biochemical behavior of variant forms of eIF-2, it will be phosphorylation at a third site cannot be ruled out, however. The phosphorylated at position 48. It is therefore highly likely that Ser48 phosphorylation is not involved in the repression of translation by DAI or HRI.

The variant 2a-AD is somewhat more highly expressed than 2a-SD (but expressed much lower than the other eIF-2α forms and is a 3.5-fold weaker inhibitor of DHFR synthesis than 2a-SD. Since neither 2a-AD nor 2a-SD is phosphorylated, it appears that the presence of Ala at position 48 decreases the inhibitory action of Asp at position 51. This suggests that 2a-AS phosphorylated on Ser51 might not repress protein synthesis as effectively as the phosphorylated wild-type protein. We speculate that Ala at residue 48 either reduces the affinity of eIF-2-GDP for eIF-2B, resulting in a failure to sequester eIF-2B, or that the variant eIF-2 has an enhanced rate of guanine nucleotide exchange that may not require catalysis by eIF-2B. To test these postulates, nearly pure variant forms of eIF-2 are required, but are not readily obtained from transiently transfected cell populations since only a minor fraction of the cells are altered. In order to test the biochemical behavior of variant forms of eIF-2, it will be necessary to purify variant eIF-2 from long term transfected cell lines overexpressing variant eIF-2α subunits, or alternatively to purify the various subunits expressed in bacteria and reconstitute variant eIF-2 complexes.

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