MOLECULAR AND CELLULAR BIOLOGY, Nov. 1991, p. 5435–5445
0270-7306/91/115435-11$02.00/0

Vol. 11, No. 11

Expression of Antisense RNA against Initiation Factor eIF-4E mRNA in HeLa Cells Results in Lengthened Cell Division Times, Diminished Translation Rates, and Reduced Levels of Both eIF-4E and the p220 Component of eIF-4F

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Received 16 April 1991/Accepted 2 August 1991

HeLa cells were transformed to express antisense RNA against initiation factor eIF-4E mRNA from an inducible promoter. In the absence of inducer, these cells (AS cells) were morphologically similar to control cells but grew four- to sevenfold more slowly. Induction of antisense RNA production was lethal. Both eIF-4E mRNA and protein levels were reduced in proportion to the degree of antisense RNA expression, as were the rates of protein synthesis in vivo and in vitro. Polysomes were disaggregated with a concomitant increase in ribosomal subunits. Translation in vitro was restored by addition of the initiation factor complex eIF-4F but not by eIF-4E alone. Immunological analysis revealed that the p220 component of eIF-4F was decreased in extracts of AS cells and undetectable in AS cells treated with inducer, suggesting that p220 and eIF-4E levels are coordinately regulated. eIF-4A, another component of eIF-4F, was unaltered.

Formation of the 48S initiation complex is an ATP-dependent process whereby mRNA becomes bound to the 43S initiation complex (3, 61). This is considered to be the rate-limiting step in protein synthesis under normal (e.g., non-virus-infected) conditions, a conclusion that is based on the observations that (i) 48S complexes are considerably less abundant than 43S complexes (11) and can usually be detected only in the presence of inhibitors (56) and (ii) the ATP-dependent step of initiation is rate limiting (42). The polypeptides which are most directly involved in binding of mRNA to the 43S complex belong to the eIF-4 group of initiation factors (reviewed in references 49 and 58): eIF-4A, a 46-kDa RNA-dependent ATPase; eIF-4B, an 80-kDa polypeptide which stimulates the activity of eIF-4A; eIF-4E, a 25-kDa cap-binding protein; and a 220-kDa component referred to as either p220 (23) or eIF-4Fγ (55). Collectively, these factors carry out the ATP-dependent unwinding of secondary structure in mRNA beginning from the 5′ terminus, which accompanies (or precedes) the migration of the 40S ribosomal subunit to the initiation codon.

eIF-4E is a phosphoprotein and exists as a mixture of phosphorylated and unphosphorylated forms in rabbit reticulocytes (51) and HeLa cells (8, 16). The overall rate of protein synthesis is correlated with the degree of eIF-4E phosphorylation in a number of systems: both are decreased in HeLa cells following heat shock (16) and during mitosis (5), and both are increased in reticulocytes stimulated with phorbol esters (47), 3T3 L1 or HIR 3.5 cells stimulated with insulin (41, 48), 3T3 fibroblasts stimulated with serum (34), B lymphocytes stimulated with bacterial lipopolysaccharide or phorbol esters (53), and epithelial cells stimulated with epidermal growth factor (15). Furthermore, whereas eIF-4E accompanies the transfer of mRNA to the 48S initiation complex (29), a variant containing Ala rather than Ser at the major phosphorylation site (designated the [Ala^{53}]eIF-4E variant) fails to be transferred (33). These observations suggest that phosphorylation of eIF-4E is obligatory for its action in the transfer of mRNA to the 43S initiation complex.

More direct evidence that eIF-4E plays an important role in controlling the overall rate of protein synthesis comes from cell transfection studies with vectors expressing eIF-4E. Overexpression of eIF-4E by three- to eightfold over endogenous levels causes shortening of cell doubling times and loss of contact inhibition of rat fibroblasts (40) and HeLa cells (12). Transfection with vectors expressing the [Ala^{53}]eIF-4E variant does not cause these effects. These results, together with the knowledge that eIF-4E is the least abundant of the initiation factors (16) and is present at only 1/10 the molar concentration of mRNA (29), implicate eIF-4E as a major determinant of the overall rate of protein synthesis.

The role of p220 in initiation is less well defined. p220 can be isolated from high-salt-treated cell lysates or ribosomal pellets in complexes with eIF-4E alone (9, 22), with eIF-4A and eIF-4E (17, 26) (in this case the complex is termed eIF-4F), with eIF-4A, eIF-4B, and eIF-4E (26), and with eIF-3 (27). Whereas eIF-4E alone was originally reported to restore protein synthesis to extracts of poliovirus-infected cells (62), subsequent studies indicated that high-molecular-weight complexes containing p220 as well as eIF-4E were required (60). An explanation for this was provided by the finding that poliovirus infection causes proteolytic cleavage of p220 (23). Buckley and Ehrenfeld (9), on the other hand, could not demonstrate the existence of a p220:eIF-4E complex in the total cytoplasmic extract of HeLa cells not treated with high salt. Thus, while it is clear that p220 is intimately involved in the formation of 48S initiation complexes involving capped mRNA, neither its mechanism of action nor the nature of its interactions with other initiation factor polypeptides or with the 40S ribosome is fully understood.

In this study, we have further explored the role of eIF-4E by expressing antisense RNA (AS RNA) against eIF-4E mRNA. The results are consistent with the idea that eIF-4E

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plays a key role in determining the overall rate of protein synthesis and cellular growth. Unexpectedly, we also found that expression of AS RNA reduced the level of p220 as well as the level of eIF-4E.

MATERIALS AND METHODS

Plasmids and oligonucleotides. The *Escherichia coli*-mammalian shuttle vector RDB, which replicates episomally, has been described previously (12, 13). RDB-0 is the vector alone, containing no inducible promoter or antisense sequences. Plasmid pG-eIF-4E is pGem-7Zf(+) (Promega Biotec) containing the human eIF-4E cDNA from plasmid pTCEEC (12).

All oligonucleotides were synthesized at the University of Kentucky Macromolecular Synthesis Facility. These are (i) antisense oligonucleotide 5′-AGTCGCCATCTTAGATCGAT-3′, complementary to nucleotides (nt) −11 to +9 of human eIF-4E mRNA (numbering system of Rychlik et al. [50]); (ii) polymerase chain reaction (PCR) 5′ primer 5′-TACACATCC CCAGAATCCATAAAT-3′, the same sense as nt 885 to 909 of eIF-4E mRNA; and (iii) PCR 3′ primer 5′-TAACCAA AGCAAAATAACCTAAGT-3′, complementary to nt 1520 to 1544 eIF-4E mRNA. Optimal annealing temperatures for PCR were calculated by using the computer program OLIGO (52). PCR conditions using these temperatures were then optimized to produce a single product.

The vector to express AS RNA against eIF-4E mRNA was constructed from RDB-DRE (13) by insertion of a double-stranded form of the antisense oligonucleotide into the *Xba*I site of the polylinker. *E. coli* JM110 cells were transformed, and plasmids from ampicillin-resistant colonies were screened for the presence of a *Cla*I site, which is created by the insertion of the antisense oligonucleotide. The fidelity of the construction and orientation of the insert were confirmed by DNA sequencing. The plasmid containing the antisense sequence in the proper orientation was called RDB-AS.

Cell culture, transfection protocols, and selection of G418-resistant clones were carried out as described previously (12). Where indicated, 10 nM tetrachlorodibenzo-*p*-dioxin (TCDD) was added to the culture medium.

Determination of RNA levels. RNA was isolated as follows. Cells were resuspended in *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid (HEPES)-buffered saline containing 2 mM EDTA. After pelleting by centrifugation at 800 × *g*, they were resuspended in 0.5 ml of lysis buffer (50 mM Tris, 0.5% sodium dodecyl sulfate [SDS], 0.1 mg of proteinase K per ml; adjusted to pH 7.2) and incubated at 37°C for 6 h. The NaCl concentration was then adjusted to 1 M, and the samples were kept on ice overnight to precipitate the bulk of the chromosomal DNA. After a 5-min centrifugation, the supernatant was phenol extracted once, and nucleic acids were precipitated and washed with 70% ethanol. Total RNA was resuspended in 50 mM Tris–2.5 mM EDTA, pH 7.2. Integrity of the RNA was assessed by denaturing agarose gel electrophoresis. For the PCR quantitations, RNA was further treated with DNase (RQI; Promega), phenol-chloroform-isoamyl alcohol extracted, and ethanol precipitated. RNA concentrations were determined spectrophotometrically.

RNase protection assay. A 771-base, minus-sense probe was generated by in vitro transcription with SP6 polymerase of pG-eIF-4E linearized at the *Acc*I site. Transcription reaction mixes contained (in a total volume of 20 μl) 40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 10 mM NaCl, 2 mM spermidine, 10 mM dithiothreitol, 0.4 mM each ATP, CTP, and GTP, 0.2 mCi of [5,6-^{3}H]UTP, and 1 μg of pG-eIF-4E. Transcription reaction mixes were incubated 1 h at 37°C and then heated to 70°C for 5 min. DNase RQ1 (5 U) was added, and the samples were incubated at 30°C for 15 min. Samples were adjusted to 10 mM EDTA and 0.2 M NaCl and extracted with phenol-chloroform, and the RNA was precipitated with 3 volumes of ethanol. The precipitate was washed once with 70% ethanol and dried. The probe thus produced had a specific activity of 2.3×10^7 cpm/μg. Test RNA was annealed to the probe (2×10^5 cpm) overnight at 50°C in 50 μl of a solution containing 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.7), 0.4 M NaCl, 1 mM EDTA, and 80% formamide. Then 0.3 ml of an ice-cold solution containing 0.3 M NaCl, 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 15 μg of RNase A (Boehringer) was added, and the solution was incubated at 30°C for 1 h. Following extraction of the sample with phenol-chloroform, 5 μg of carrier yeast tRNA was added, and nucleic acids were precipitated with 2 volumes of ethanol.

PCR amplification. One to five micrograms of total RNA was reverse transcribed as described by Wang et al. (64), with minor modifications. A 10-μl reverse transcription reaction mix contained 20 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM $MgCl_2$, 100 μg of bovine serum albumin per ml, 1 mM dithiothreitol, 50 μM each deoxynucleoside triphosphate, 5 to 10 U of RNasin (RNA Guard; Promega), 0.1 μg of the 3′ primer, and 3 U of avian myeloblastosis virus reverse transcriptase (Promega). Prior to the addition of the other components, the RNA and primer were heated at 65°C for 10 min in diethylpyrocarbonate-treated H_2O. Reactions were carried out at 37°C for 1 h. Aliquots of the reverse transcriptase reaction were combined with carrier rRNA to produce a total of 0.05 to 0.4 μg of total RNA and were amplified in a 100-μl reaction mix containing 2 U of *Taq* DNA polymerase (AmpliTaq; Perkin-Elmer-Cetus Corp.), 0.3 μg of each primer, 200 μM deoxynucleoside triphosphates, and 1 to 10 μCi of [α-^{32}P]dATP. The PCR reaction was carried out in a Perkin-Elmer-Cetus thermocycler at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Aliquots of 1 to 3 μl were taken at selected points by pausing the machine for 1 min following the 72°C step, spotted onto Whatman 3MM filter disks, and batch washed in trichloroacetic acid (TCA); the radioactivity was determined in toluene-based scintillation fluid.

Northern (RNA) blots. Actin mRNA was detected in total RNA by Northern analysis (13), using human β-actin cDNA in pGEM3 (1.95×10^8 cpm/μg) as the probe. AS RNA was similarly detected with BS-DRE (13) (2.2×10^8 cpm/μg) as the probe. The relative abundances of actin mRNA and AS RNA were calculated from the specific activities of the two probes and the Northern results shown in Fig. 1B (inset) and Fig. 4C. The ratio of the AS RNA signal from AS RNA-expressing HeLa cells (AS cells) in the absence of inducer to that of actin mRNA was 0.022, whereas that from the cells plus inducer was 0.061.

Immunologic analysis. Extracts from HeLa and AS cells were prepared as described previously (12). The protein concentration in each sample was determined by the method of Bradford (6), and equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis (PAGE) using an 8% gel. The proteins were transferred to a polyvinylidene difluoride membrane (MSI Nitroplus 2000; Micron Separations Inc., Westboro, Mass.) and probed with a monoclonal antibody against p220 (19; gift from Diane Etchison) or eIF-4A (17; gift from Hans Trachsel) as described by Winston (66). The secondary antibody was affinity-purified

horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G (Vector Laboratories, Burlingame, Calif.). The antigen-antibody complex was visualized by using the chromogenic substrate 3,3′-diaminobenzidine hydrochloride.

Measurement of protein synthesis. In vivo protein synthesis rates were measured by incorporating [3,4,5-^{3}H]leucine at 30 μCi/ml into cells for 3 h. A cytoplasmic extract was prepared and subjected to either SDS-PAGE or TCA precipitation. The specific radioactivity of the leucine pool was determined by extracting the TCA-soluble fraction with 5 volumes of ether and subjecting equal aliquots to scintillation spectrometry and automated amino acid analysis in the University of Kentucky Macromolecular Structure Analysis Facility.

In vitro protein synthesis was performed as follows. Cells were collected by centrifugation and washed once with phosphate-buffered saline (PBS). The pelleted cells were homogenized in 2.5 volumes of hypotonic buffer containing 20 mM HEPES (pH 7.4), 10 mM NaCl, 1.5 mM magnesium acetate, 0.3% Brij 58, 300 U of RNasin per ml, 2 μg of leupeptin and pepstatin per ml, and 50 μM hemin. Cytoplasmic extracts were obtained by sedimenting nuclei and cell debris at 30,000 × *g*. Protein synthesis was carried out at 30°C for 1 h in 50-μl reaction mixes containing 30 μl of cell extract, 10 μl of reaction mix (0.15 M NaCl, 0.2 M potassium acetate, 5 mM dithiothreitol, 74 mM creatine phosphate, 40 U of creatine phosphokinase per ml, 1 mM ATP, 1 mM GTP, 0.1 M HEPES, pH 7.4), 35 μM amino acids minus leucine, and 50 μCi of [3,4,5-^{3}H]leucine (NEN).

Polysome analysis. The protocol of White et al. (65) was carried out with the following modifications: (i) the cells were detached by using a solution of 0.05% (wt/vol) trypsin and 0.53 mM EDTA and washed with PBS, (ii) no NaCl was added during purification of the cytoplasmic extracts, and (iii) the cytoplasmic extracts were layered onto linear 4.5-ml sucrose gradients (0.5 to 1.5 M), which were centrifuged for 50 min at 4°C in a SW60Ti rotor at 50,000 rpm.

Protein preparations. eIF-4E was purified from human erythrocytes as described by Rychlik et al. (51) and from HeLa cells as described by De Benedetti and Rhoads (12). eIF-4F was purified from rabbit reticulocyte ribosomal salt wash by m^{7}GTP-Sepharose chromatography followed by Mono Q chromatography as described by Lamphear and Panniers (39).

RESULTS

Phenotype of transfected cells. The vector used to express AS RNA was derived from BK virus and pSV-2neo and contained a mouse mammary tumor virus (MMTV) long terminal repeat promoter linked to a dioxin-responsive enhancer (13). A sequence complementary to 20 nt near the 5′ terminus of eIF-4E mRNA was placed under control of the inducible promoter. Cells harboring this vector are resistant to the antibiotic G418 as a result of the expression of the aminoglycoside phosphotransferase gene present in the construct. Cells transformed with this vector (hereafter referred to as AS cells) grew slowly, with a doubling time of about 100 h (Fig. 1A), whereas untransfected HeLa cells doubled in approximately 25 h (Fig. 1B). The fact that inhibitory effects were observed in the absence of inducer is consistent with previous observations that a low level of constitutive gene expression occurs with this promoter-enhancer combination (12, 13, 32). Addition of the inducer TCDD to AS cells caused further slowing of the growth rate and then a decline in cell number after 2 days (Fig. 1A). TCDD had no detectable effect on untransfected HeLa cells (Fig. 1B) or on cells transformed with RDB-0 (data not shown).

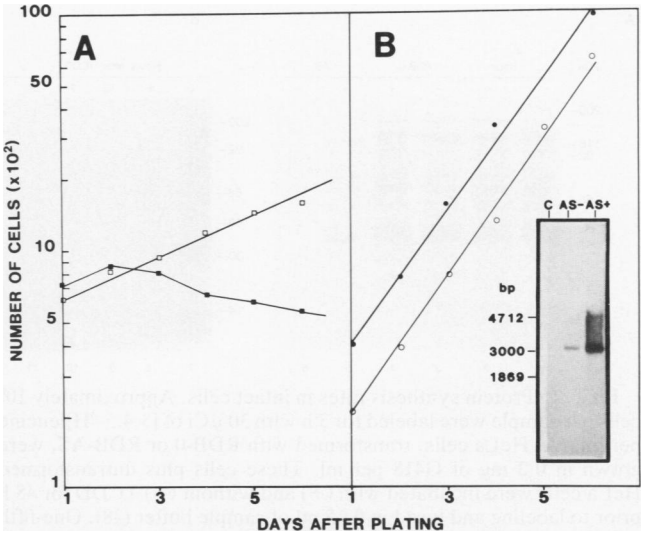

FIG. 1. Growth curves of HeLa and AS cells. Cells were plated in 25-cm^2 flasks with a graduated bottom. The average cell number in four random 1-cm^2 grids was taken each day, beginning 1 day after plating. (A) AS cells cultured in 0.2 mg of G418 per ml with (closed squares) and without (open squares) the inducer TCDD. (B) Control untransfected HeLa cells grown with (open circles) and without (closed circles) TCDD. (Inset) Northern analysis of antisense RNA produced in control HeLa cells (C), AS cells without inducer (AS−), and AS cells with inducer for 18 h (AS+). Total RNA was probed with BS-DRE (see Materials and Methods). The film was exposed for 21 days.

The copy number of this vector can be altered by changing the concentration of G418 in the culture medium (13). The 100-h doubling time in Fig. 1A was obtained with G418 at 0.2 mg/ml. When cells were cultured in G418 at 0.4 or 0.6 mg/ml, the doubling time increased to 170 h (data not shown). Conversely, when AS cells were maintained without G418 selection, they resumed normal growth rates in about 2 weeks, presumably because of a reduction of vector copies. HeLa cells transformed with RDB-0 grew at normal rates in the presence of G418 at either 0.2 or 0.4 mg/ml (data not shown). These results indicate that the phenotype of slow growth is due to the expression of eIF-4E antisense sequences and not to the vector per se, G418 or TCDD.

The nature of the RNA product containing the AS sequence was examined by Northern hybridization (Fig. 1B, inset). The predominant product was approximately 3 kb, consistent with the RNA beginning with the MMTV promoter and ending with the termination signal and poly(A) addition site of BK virus. The RNA downstream of the AS sequence is derived from Bluescript. Control HeLa cells contained no cross-hybridizing RNAs (lane C). The major AS RNA was present at 2.2% the molar concentration of β-actin mRNA in uninduced cells (lane AS−), and this increased to 6.1% after 18 h of induction (lane AS+).

In vivo protein synthesis. If AS RNA decreases the intracellular levels of eIF-4E, and if eIF-4E is rate limiting for protein synthesis, then the slow growth of AS cells could be due to a reduction in overall protein synthesis rate. To test this, we measured the incorporation of [^{3}H]leucine into protein for 6 h in control and AS cells, the latter grown in G418 at 0.6 mg/ml. The rate of leucine incorporation was

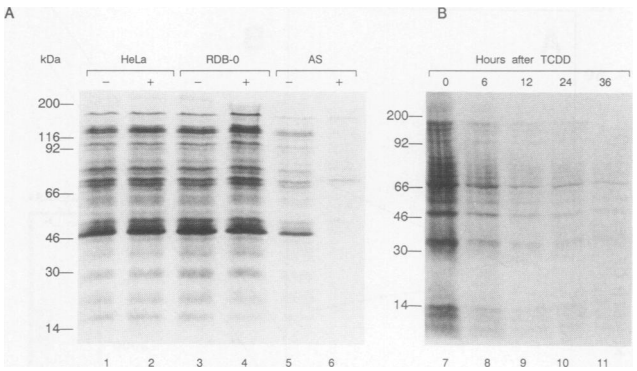

FIG. 2. Protein synthesis rates in intact cells. Approximately 10^6 cells per sample were labeled for 3 h with 30 μCi of [3,4,5-^{3}H]leucine per ml. (A) HeLa cells, transformed with RDB-0 or RDB-AS, were grown in 0.2 mg of G418 per ml. These cells plus untransformed HeLa cells were incubated with (+) and without (−) TCDD for 48 h prior to labeling and lysed in 0.15 ml of sample buffer (38). One-fifth of the sample was subjected to SDS-PAGE on 10% gels. (B) AS cells were labeled with [3,4,5-^{3}H]leucine for 3 h at the times indicated following the addition of TCDD. The positions of molecular weight standards are indicated. The TCA-precipitable radioactivities of selected samples were 26,285 (lane 3), 9,385 (lane 5), 2,165 (lane 6), 10,775 (lane 7), 7,040 (lane 8), 5,985 (lane 9), 3730 (lane 10), and 2,980 (lane 11) cpm/μl. The film for panel B was exposed three times longer than that for panel A.

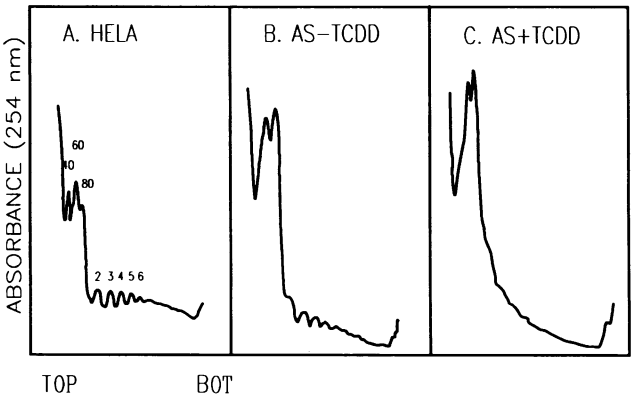

FIG. 3. Distribution of polysomes after AS RNA expression. Untransfected HeLa cells (A) and AS cells maintained in 0.2 mg of G418 per ml without (B) or with (C) treatment for 42 h with TCDD were harvested and analyzed for polysomes as described in Materials and Methods. Ribosomal subunits and monosomes are indicated by 40, 60, and 80. Disomes, trisomes, etc., are indicated by 2, 3, etc. The direction of sedimentation in each case was left to right.

reduced by 10-fold in AS cells (10,325 versus 1,230 cpm/10^4 cells). Addition of TCDD at the beginning of the 6-h labeling period did not change the rate of incorporation in either control or AS cells, suggesting that the inducer requires a period of time to produce its effect.

In a second experiment, AS and control RDB-0 cells were maintained in 0.2 mg of G418 per ml to produce a lower copy number of the vector. The transfected cells as well as untransfected HeLa cells were then incubated with and without TCDD, in this case 48 h prior to labeling with [^{3}H]leucine for 3 h (Fig. 2). The leucine pool-specific radioactivities of the various cell lines, with or without TCDD treatment, were the same to within 6%. Thus, the intensity of bands in Fig. 2 is proportional to the protein synthesis rate. The overall rate of protein synthesis was reduced 2.8-fold in the AS cells (Fig. 2A, lane 5 versus lane 3), and the addition of TCDD caused a further 4.3-fold decrease (lane 6). Protein synthesis was the same in HeLa and RDB-0 cells, and TCDD had no effect (lanes 1 to 4). Synthesis of most proteins was reduced in AS cells, but that of some proteins was more resistant to the general inhibition. These presumably result from the translation of "strong" mRNAs, i.e., those having the least requirement for eIF-4 group initiation factors (reviewed in reference 41).

We also studied the time course of TCDD action on the rate of protein synthesis in AS cells. The addition of TCDD produced a progressive decrease in protein synthesis rates over a 36-h period (Fig. 2B). Taken together, these experiments indicate that the expression of eIF-4E AS RNA, whether determined by vector copy number or by induction of the promoter, reduces the in vivo rate of protein synthesis in a dose- and time-dependent manner.

If the depletion of eIF-4E is responsible for the observed inhibition of protein synthesis, one would expect polysomes to be disaggregated because eIF-4E acts at the step wherein mRNA becomes bound to the ribosome. To test this prediction, polysome profiles were measured in control HeLa cells and AS cells in the presence and absence of TCDD (Fig. 3). The results indicated that polysomes were decreased in AS cells with a corresponding increase in ribosomal subunits (Fig. 3B versus 3A), and when TCDD was added to the medium, polysomes became undetectable (Fig. 3C).

eIF-4E mRNA levels. AS RNA is believed to exert its effect on target gene expression by inhibiting the synthesis, processing, transport, or translation of a specific mRNA (24). To distinguish between translational and nontranslational effects in the case of eIF-4E AS RNA, we measured the level of eIF-4E mRNA. Cells were treated with and without TCDD for 36 h, a period which does not result in cell death or even in the complete arrest of cell growth (Fig. 1A), and the relative levels of eIF-4E mRNA were determined by two independent methods. In the first, an RNase protection assay, a minus-sense probe was generated by in vitro transcription of the cDNA for eIF-4E with SP6 RNA polymerase (Fig. 4A; Fig. 4B, lane 1; note that the markers refer to mobility of double-stranded DNA and do not apply to the single-stranded RNA probe). RNase A completely digested the probe in the absence of complementary mRNA (lane 2). Hybridization of the probe to a full-length, plus-sense transcript, produced by transcription of the same plasmid with T7 RNA polymerase, resulted in a protected fragment migrating at 725 bp (lane 3), the expected position of the duplex RNA. Total RNA extracted from control cells yielded a strong band (lane 4). AS cells, by contrast, yielded considerably less of the protected band (lane 5), and TCDD treatment further reduced this level (lane 6). Bands corresponding to the protected probe were excised, and the radioactivity was determined by scintillation spectrometry (Fig. 4B, bottom). From the radioactivity in lanes 3 and 4, we calculate that eIF-4E mRNA represents 0.04% of total HeLa cell mRNA (see figure legend). AS cells and AS cells treated with TCDD contained 3- and 11-fold, respectively, less eIF-4E mRNA than did control cells. As a control that all mRNA species were not decreased by AS RNA expression, we also measured actin mRNA content during the course of TCDD induction (Fig. 4C). Actin mRNA levels did not change significantly over the 48-h period tested.

The second method used for mRNA quantitation was

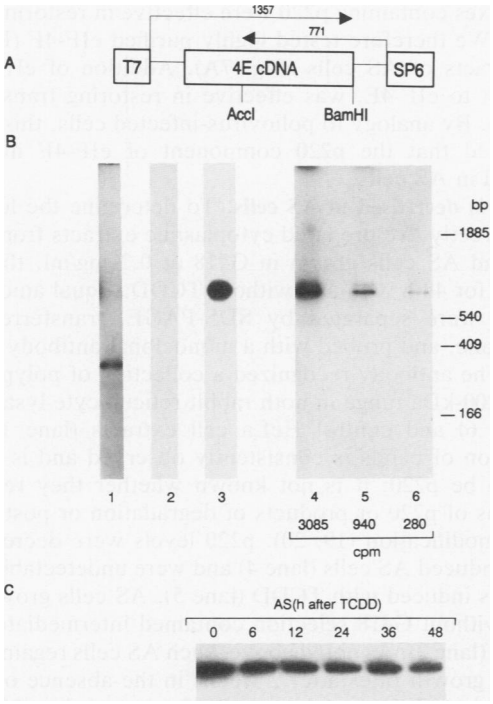

FIG. 4. Quantitation of eIF-4E mRNA by RNase protection. (A) Transcription template used to synthesize the eIF-4E mRNA plus- and minus-sense RNAs. (B) RNase protection assays carried out as described in Materials and Methods. After ethanol precipitation of the protected RNA, the samples were resuspended in 10 mM Tris-HCl (pH 7.2)–1 mM EDTA–15% glycerol and separated on a nondenaturing 1.5% agarose gel, using *Dde*I-cut pGem-7Zf(+) DNA as markers. In this system, RNA duplexes shorter than 1.5 kb comigrate with DNA duplexes of the same size. Lane 1, 500 cpm of single-stranded probe (synthesized with SP6 polymerase); lane 2, probe digested with RNase A in the absence of complementary RNA; lane 3, signal obtained after annealing of probe to 5 ng of plus-sense RNA (synthesized with T7 polymerase). Total RNA (70 μg) from control untransfected cells (lane 4), AS cells grown in 0.2 mg of G418 per ml (lane 5), and AS cells treated for 48 h with TCDD (lane 6) was annealed to the probe and digested with RNase A. The radioactivity in the major band of selected lanes is shown at the bottom. The level of eIF-4E mRNA in control cells was estimated as follows. Five nanograms of the T7 transcript protected 27,930 cpm of the probe. Assuming that 2% of total RNA is mRNA, the 70 μg of total RNA used in protection assays contained 1,400 ng of total mRNA, and this protected 3,085 cpm of probe. 3,085/27,930 × 5 ng = 0.55 ng of eIF-4E mRNA. 0.55/1,400 = 0.0004. Thus, eIF-4E mRNA represents 0.04% of total HeLa mRNA. (C) Northern analysis of actin mRNA. Total RNA was extracted from AS cells at various times after addition of TCDD as indicated and analyzed by Northern blotting using a β-actin cDNA probe (see Materials and Methods). The film was exposed for 1 day.

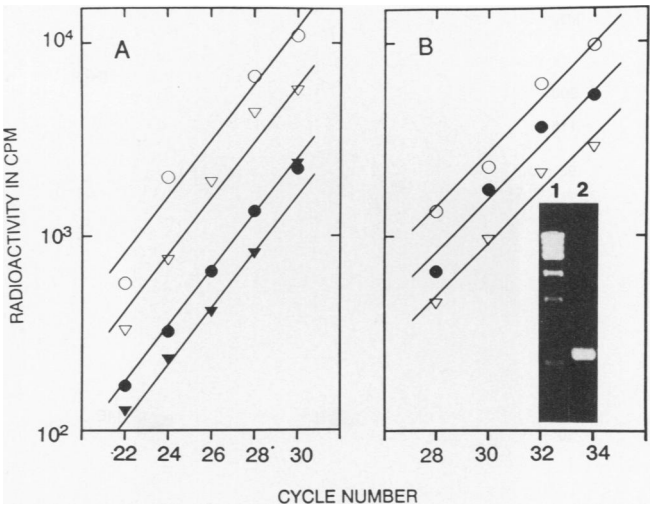

FIG. 5. Quantitation of eIF-4E mRNA in control and AS cells by PCR. (A) Dose response quantitation of eIF-4E mRNA in total RNA isolated from continuous rat embryo fibroblast cells. The amounts of total RNA used for PCR were 0.4 μg (open circles), 0.2 μg (open triangles), 0.1 μg (closed circles), and 0.05 μg (closed triangles). Carrier RNA was added after reverse transcription, and the samples were subjected to PCR amplification in the presence of [α-^{32}P]dATP, using eIF-4E mRNA-specific primers. Products were quantitated by TCA precipitation and scintillation spectrometry. (B) Effect of AS RNA on eIF-4E mRNA levels. Total RNA (0.1 μg) from untransfected HeLa cells (open circles), AS cells (closed circles), and AS cells treated with TCDD as for Fig. 3 (open triangles) was analyzed as for panel A. (Inset) Ethidium bromide-stained 2.5% agarose gel of *Hin*dIII-cut λ DNA (lane 1) and the eIF-4E mRNA-specific PCR product (lane 2).

selective reverse transcription of eIF-4E mRNA followed by PCR in the presence of [α-^{32}P]dATP. This yielded a single PCR product of 659 bp which could be quantitated directly by precipitation with TCA and scintillation spectrometry (Fig. 5B, inset). To demonstrate that the method was quantitative, we subjected several dilutions of reverse-transcribed RNA from a single source to PCR (Fig. 5A). The product increased exponentially for each dilution and was proportional to the amount of RNA added, provided that it was measured in the exponential range. AS cells contained 1.7-fold less eIF-4E mRNA than did control HeLa cells by this assay, and AS cells treated with TCDD for 36 h contained 3-fold less than did control cells (Fig. 5B). The quantitative discrepancy between the two methods is not understood, but it is clear that eIF-4E mRNA was significantly reduced in AS cells and that a further decrease occurred upon induction of the promoter with TCDD.

eIF-4E protein levels. The level of the eIF-4E protein was measured in cytoplasmic extracts from control HeLa and AS cells grown in 0.6 mg of G418 per ml with or without a 48-h induction with TCDD. eIF-4E was affinity purified on m^{7}GTP-Sepharose columns, and aliquots of both total protein and column-bound protein were separated by SDS-PAGE and stained with Coomassie blue (Fig. 6). Total protein patterns were similar (lanes 1 to 3), but eIF-4E was detectable only in the column-bound fraction of control cells (lane 4 versus lanes 5 and 6). In a similar experiment, the vector copy number was decreased by growing the cells in 0.2 mg of G418 per ml in an attempt to obtain a less severe reduction of eIF-4E. Under these conditions, AS cells contained sevenfold less eIF-4E than did control cells, as estimated by densitometry, whereas eIF-4E could not be detected in TCDD-treated AS cells (data not shown). Interestingly, an m^{7}GTP-Sepharose-enriched protein of 220 kDa was also decreased in AS compared with control cell extracts (lane 4 versus lanes 5 and 6). On the basis of its molecular weight and retention on m^{7}GTP-Sepharose, this is likely to be the p220 component of eIF-4F (17, 26, 60).

In vitro protein synthesis. The results presented thus far indicate that AS cells grow slowly, that protein synthesis in vivo is decreased up to 10-fold, and that the cellular levels of both eIF-4E mRNA and eIF-4E protein are decreased in

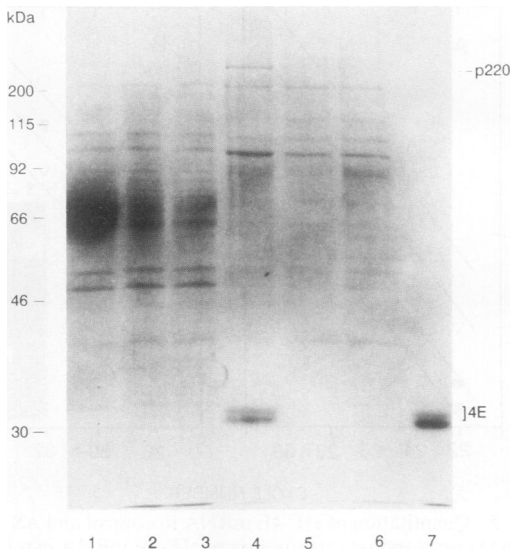

FIG. 6. Quantitation of eIF-4E in control and AS cells. The cytoplasmic extract from 5×10^7 cells (3 ml) was applied to a 2-ml m^7GTP-Sepharose column, and the bound protein was eluted with m^7GTP and precipitated with TCA (12). The protein was resuspended in Laemmli sample buffer, subjected to SDS-PAGE, and stained with Coomassie blue. The positions of molecular weight markers, eIF-4E, and a 220-kDa polypeptide, which is thought to be the p220 component of eIF-4F, are shown. AS cells were grown in the presence of 0.6 mg of G418 per ml, and the gel used was 10%. Lanes: 1 to 3, 30 μl of total protein from control HeLa cells, AS cells, and AS cells treated for 48 h with TCDD, respectively; 4 to 6, m^7GTP-Sepharose-bound protein from the same extracts (the entire sample); 7, 1.7 μg of purified eIF-4E from human erythrocytes. eIF-4E migrates as doublet when incompletely reduced (51). The dark bands at around 66 kDa in lanes 1 and 2 are bovine serum albumin, which was not completely removed when the cells were collected.

proportion to the expression of AS RNA. The most straightforward interpretation of these findings is that cell growth is slowed because of a decrease in protein synthesis resulting from the loss of eIF-4E. If so, then extracts of AS cells should likewise be restricted in protein synthetic capacity, and this should be restored with exogenous eIF-4E. Cell extracts were prepared from control and AS cells and assayed for [3,4,5-^{3}H]leucine incorporation. As observed for intact cells (Fig. 2), protein synthesis in extracts of AS cells was drastically reduced (Fig. 7A, lane 2 versus lane 1). Surprisingly, however, the addition of purified eIF-4E did not stimulate translation to any significant degree (Fig. 7B, lanes 8 to 12; note that the aliquots analyzed in lanes 8 to 12 were three times larger than that in lane 7). The highest level of eIF-4E added (lane 8) corresponded to fivefold more than was present endogenously in the cell-free system from control cells (see figure legend). It was unlikely that the lack of stimulation was due to loss of activity of eIF-4E itself, since eIF-4E purified by m^7GTP-Sepharose retains its activities of cap binding, association with the 48S initiation complex (33), and restoration of translation in m^7GTP-inhibited lysates (28).

The failure of eIF-4E to stimulate translation was unexpected, since the AS RNA was targeted specifically to eIF-4E mRNA. However, this is reminiscent of the inability of eIF-4E to restore translation to extracts of poliovirus-infected cells (60). In the latter case, high-molecular-weight complexes containing p220 were effective in restoring translation. We therefore tested highly purified eIF-4F (Fig. 7C) on extracts of AS cells (Fig. 7A). Addition of eIF-4F, in contrast to eIF-4E, was effective in restoring translational activity. By analogy to poliovirus-infected cells, this finding suggested that the p220 component of eIF-4F might be affected in AS cells.

p220 is decreased in AS cells. To determine the levels of p220 directly, we prepared cytoplasmic extracts from HeLa cells and AS cells grown in G418 at 0.2 mg/ml, the latter treated for 48 h with and without TCDD. Equal amounts of protein were separated by SDS-PAGE, transferred to a membrane, and probed with a monoclonal antibody against p220. The antibody recognized a collection of polypeptides in the 200-kDa range in both rabbit reticulocyte lysate (Fig. 8, lane 6) and control HeLa cell extracts (lane 1). This collection of bands is consistently observed and is considered to be p220; it is not known whether they represent isoforms of p220 or products of degradation or posttranslational modification (19, 20). p220 levels were decreased in the uninduced AS cells (lane 4) and were undetectable in the AS cells induced with TCDD (lane 5). AS cells grown for 1 week without G418 selection contained intermediate levels of p220 (lane 2). As noted above, such AS cells regain almost normal growth rates after 2 weeks in the absence of G418. Addition of TCDD to these cells decreased the p220 level only slightly (lane 3). Presumably, the recovery of normal growth rates is due to the loss of vector, so that TCDD addition results in insufficient AS RNA to produce a major effect. We did not observe the breakdown products of p220 (100 to 130 kDa) which are characteristic of poliovirus-infected cell extracts (23), though the monoclonal antibody used is capable of recognizing such products (19).

Decay of eIF-4E, p220, and protein synthesis rates after induction with TCDD. To analyze the relationship between the levels of eIF-4E and p220 and the overall in vivo rate of protein synthesis, we determined all three parameters in a single experiment as a function of time after addition of TCDD (Fig. 9). AS cells grown in 0.2 mg of G418 per ml were treated with TCDD, and aliquots from multiwell flasks were removed at intervals. Protein synthesis was determined by pulse-labeling cells during the last 3 h of each time point. A parallel set of cells which had been previously labeled to equilibrium with [3,4,5-^{3}H]leucine was used for quantitation of eIF-4E levels. Another set was analyzed by Western immunoblotting to measure p220 levels. The results indicated that except for a slight initial lag, eIF-4E and p220 decayed with nearly the same kinetics. Protein synthesis decreased the most between 6 and 18 h, in parallel with eIF-4E and p220, and thereafter more slowly, presumably reflecting the residual translation of specific "strong" mRNAs.

Another component of the eIF-4F complex is eIF-4A, although the major portion of this factor exists in the free form (see the introduction). It was therefore of interest to determine whether eIF-4A levels were also decreased when AS RNA was expressed. eIF-4A was monitored immunologically during the course of TCDD induction (Fig. 9, double triangles). Interestingly, over the period in which eIF-4E and p220 decreased to undetectable levels, eIF-4A was unchanged. In a separate experiment, the level of eIF-4A in control HeLa cells was found to be the same as in AS cells (data not shown).

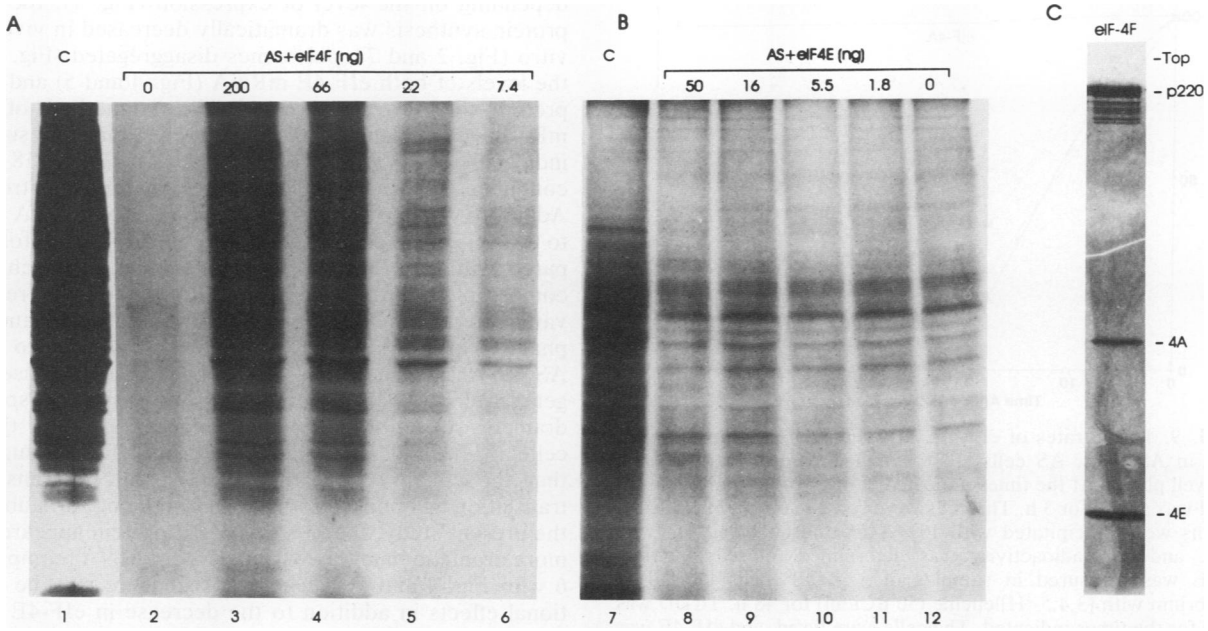

FIG. 7. Effect of added eIF-4F and eIF-4E on translation by extracts of AS cells. Proteins were labeled with [3,4,5-^{3}H]leucine in extracts of control HeLa cells (C; lanes 1 and 7) and AS cells grown in 0.6 mg/ml G418 (AS), separated by SDS-PAGE, and visualized by fluorography. (A) The indicated amounts of rabbit reticulocyte eIF-4F were added to 50-μl translation reactions. Aliquots of 5 μl were loaded on the gel. (B) Same as panel A except that human erythrocyte eIF-4E was added to translation reaction mixes. The aliquot loaded for control cell extracts (lane 7) was 5 μl, whereas that for AS extracts (lanes 8 to 12) was 15 μl. From the data in Fig. 6, we estimate that 1.0 μg of eIF-4E is present in 3 ml of HeLa extract. Thus, the control in vitro translation system (lane 7) contained 10 ng of endogenous eIF-4E in a 50-μl reaction mixture. (C) SDS-PAGE of the eIF-4F preparation used in panel A. Staining was with silver.

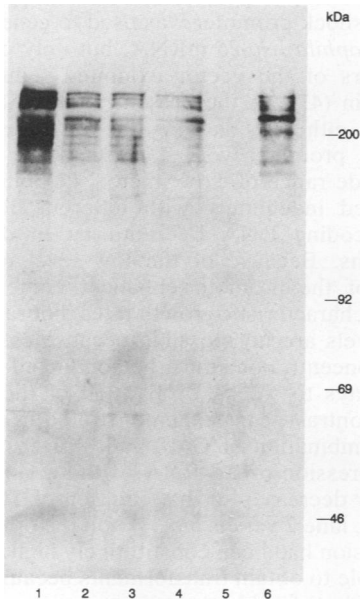

FIG. 8. Immunological analysis of p220 in control and AS cells. Cell extracts were prepared from HeLa and AS cells, and 150 μg of protein from each sample was analyzed by immunoblotting with an anti-p220 antibody. Lanes: 1, control HeLa cells; 2, AS cells cultured without G418 for 1 week; 3, same as lane 2 but treated with TCDD for 48 h; 4, AS cells cultured in 0.2 mg of G418 per ml; 5, same as lane 4 but treated with TCDD for 48 h; 6, 5 μl of rabbit reticulocyte lysate.

DISCUSSION

Determining the mechanisms which regulate the overall rate of protein synthesis is central to a general understanding of cellular metabolism. Nearly all of the translational control mechanisms identified to date occur at the initiation step, so it is logical to study the initiation factors as targets and mediators of translational regulation. Most or all of the initiation factors have now been isolated and characterized in vitro. However, the sheer complexity of protein synthesis, involving the coordinated action of over 200 proteins and 100 RNAs, requires that the roles of initiation factors be defined using in vivo systems as well. The favorable genetic approaches available in *Saccharomyces cerevisiae* have permitted several initiation factors to be studied in vivo. eIF-2β was shown to be essential to the cell and to play a role in initiation codon selection (14). Similarly, an intact eIF-4A gene is required for survival of the yeast cell (4). The eIF-4E gene is likewise essential to yeast cells (1) and, interestingly, is the same as a gene involved in control of cell division, *cdc-33* (7). The phenotype of *cdc-33* mutants resembles cells arrested at the G_0/G_1 boundary during amino acid starvation.

In higher eukaryotes, studies of initiation factors involving disruption of genes or their replacement with mutated derivatives are not presently feasible. Alternative approaches for studying these factors in vivo include (i) expressing additional copies of the initiation factor, either of the normal sequence or of a variant, by transfecting cells with expression vectors and (ii) reducing the endogenous levels of the initiation factor with antisense sequences. The first of these approaches has been used for eIF-2α, whereby the essential role of Ser-51 in regulating this factor's activity was demon-

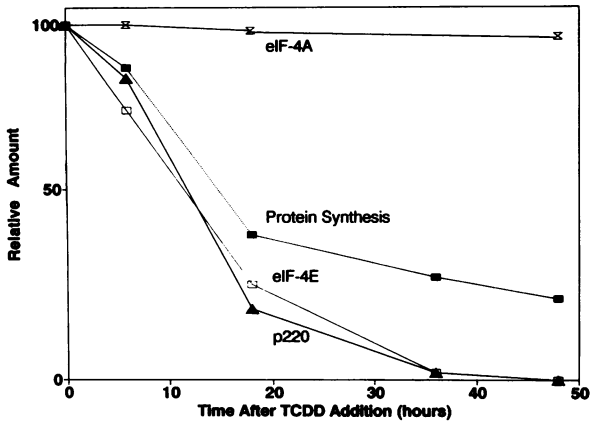

FIG. 9. Decay rates of eIF-4A, eIF-4E, p220, and protein synthesis in AS cells. AS cells (10^5) were incubated with TCDD in multiwell plates. At the times indicated, 15 μCi of [3,4,5-^{3}H]leucine per ml was added for 3 h. The cells were lysed in 0.5 ml of 1% SDS, proteins were precipitated with 10% TCA and collected on GFA filters, and the radioactivity was determined (closed squares). eIF-4E was measured in samples of 3×10^6 cells labeled to equilibrium with [3,4,5-^{3}H]leucine (50 μCi/ml) for 48 h. TCDD was added for the times indicated. The cells were lysed, and eIF-4E was isolated as described in Materials and Methods and separated on an SDS–10% polyacrylamide gel. After fluorography, the eIF-4E band was quantitated with a laser densitometer (open squares). For determination of p220 and eIF-4A, 150 μg of protein for each sample was analyzed by Western blotting as for Fig. 7. The eIF-4A (double triangles) and major p220 (closed triangles) bands were quantitated by densitometry with reflective illumination. All values are expressed relative to those for AS cells not treated with TCDD.

strated (35). The importance of the hypusine modification at Lys-50 of eIF-4D was similarly demonstrated in COS-1 cells (57). Overexpressing eIF-4B in COS-1 cells by 50-fold resulted in a general inhibition of translation (45). This approach has also been used for eIF-4E. Overexpression by as little as three- to eightfold led to a rapid growth phenotype reminiscent of oncogenically transformed cells (12, 40). The absence of this phenotype in cells transformed with the same vectors expressing the [Ala^{53}]eIF-4E variant provides in vivo evidence that phosphorylation at Ser-53 is essential for eIF-4E activity.

The second approach, to reduce the level of an initiation factor with AS RNA technology, has not been used prior to the study reported here. AS RNA is thought to inhibit expression of an mRNA by the formation of an RNA duplex which either inhibits its processing and transport from the nucleus, prevents its translation, or enhances its degradation (24). Melton (44) used AS RNA in *Xenopus* oocytes to inhibit β-globin translation and found that sequences complementary to the 5′ noncoding region or the 5′ region including the translation initiation site were more inhibitory than those complementary to the 3′ coding or 3′ noncoding regions. Izant and Weintraub (31) observed maximal inhibition of thymidine kinase expression in LTK$^-$ cells when they used AS RNA directed against the 5′ untranslated portion of the mRNA. On the basis of these results, we constructed the vector to express RNA which was complementary to 20 nt at the 5′ terminus of eIF-4E mRNA.

Whether because of the level of AS RNA in the cell or because of the site of complementarity, the expression of AS RNA against eIF-4E mRNA produced marked effects in our system. The rate of cell division was slowed or stopped, depending on the level of expression (Fig. 1), the rate of protein synthesis was dramatically decreased in vivo and in vitro (Fig. 2 and 7), polysomes disaggregated (Fig. 3), and the levels of both eIF-4E mRNA (Fig. 4 and 5) and eIF-4E protein (Fig. 6) were decreased. The two methods of eIF-4E mRNA estimation gave qualitatively similar results and indicated that AS cells grown in 0.2 mg of G418 per ml contained 1.7- to 3-fold less mRNA than did control cells. Addition of the inducer further reduced the mRNA level 2- to 3-fold, giving an overall reduction of 3- to 10-fold compared with control cells. Contente et al. (10) achieved a comparable reduction of *rrg* mRNA, but the reduction varied greatly among clonal transformants. Griep and Westphal (25) used the simian virus 40 early promoter to express AS RNA complementary to portions of the mouse c-*myc* gene. Cells transformed with these constructs displayed a dramatic reduction in *myc* protein. Surprisingly, the total cellular levels of *myc* mRNA were not affected, suggesting that the effect was at the level of mRNA transport or translation as opposed to synthesis, splicing, or stability. In the present study, the loss of eIF-4E protein appeared to be more dramatic than the loss of eIF-4E mRNA (compare Fig. 6 with Fig. 4 and 5), suggesting that there may be translational effects in addition to the decrease in eIF-4E mRNA levels.

Another important feature of the system described here is the ability to regulate the level of AS RNA expression. Inducible promoters have been used in previous studies to express AS RNA with varying success. Holt et al. (30) made constructs expressing c-*fos* AS RNA under the control of the MMTV promoter. Addition of dexamethasone to stable transformants caused rapid production of AS RNA and inhibition of cell proliferation. On the other hand, AS RNA against the mRNA encoding the tissue inhibitor of metalloproteinases, directed from a metallothionine promoter, was found to be constitutive and not inducible by heavy metals (36). A heat shock promoter was used to generate AS RNA against *Drosophila hsp-26* mRNA, but only cells with high copy numbers of the vector exhibited reduced levels of *hsp-26* protein (43). In the present study, AS RNA levels were altered either by varying the G418 selection or by inducing the promoter with TCDD. These two methods provide a wide range of experimental flexibility. Cells may be maintained indefinitely with different dosages of the antisense-encoding DNA by culturing in different G418 concentrations. Because of the low level of constitutive expression of the promoter-enhancer combination, these cells exhibit characteristic growth rates, but rapid changes in AS RNA levels are not possible because establishment of new G418 concentrations must be conducted in stages over periods of days to weeks. Activation of the promoter by TCDD, by contrast, can be demonstrated in as little as 1 h (18). The combination of G418 and TCDD is required for maximal expression of AS RNA; without G418, the vector copy number decreases so that addition of TCDD has little effect (Fig. 8, lane 2 versus lane 3). On the other hand, if AS RNA expression had been constitutively high, we would not have been able to obtain transformants because of the lethal phenotype (Fig. 1A, closed squares).

The coordinate loss of eIF-4E and p220 in AS cells was unexpected and may open the way for studies to clarify the relationship between these two polypeptides. Originally, eIF-4E was independently detected as a polypeptide which would either cross-link to mRNA caps (59), reverse the inhibition of protein synthesis caused by poliovirus infection (62), or reverse the inhibition of cell-free translation caused

by cap analogs (28). Subsequently, Tahara et al. (60) found that high-molecular-weight complexes containing eIF-4E were effective in reversing poliovirus inhibition but that eIF-4E alone was not. p220 was first identified as a protein which was proteolytically cleaved coincident with the shutoff of protein synthesis caused by poliovirus infection (23). It was then found by Grifo et al. (26) and Edery et al. (17) to be a component of the high-molecular-weight complexes discovered by Tahara et al. (60). It is worth noting, however, that all such complexes have been isolated from cells or ribosomal extracts after treatment with 0.5 M KCl, and that attempts to isolate p220:eIF-4E complexes from whole cell lysates without high-salt treatment have failed (9). Thus, the association between these two proteins may be transient and occur only during a specific stage of initiation. The phosphorylation of eIF-4E may also have a bearing on the association of p220 and eIF-4E. The nonphosphorylatable [Ala^{53}]eIF-4E variant does not become bound to the 48S initiation complex (33). Assuming that eIF-4E is bound to the 48S complex via p220, this may mean that phosphorylated eIF-4E has a higher affinity for p220 than does unphosphorylated eIF-4E. Consistent with this idea, Tuazon et al. (63) found that phosphorylated eIF-4E could be isolated in a complex with p220, but that a similar complex was not formed when unphosphorylated eIF-4E was added. This would be reminiscent of eIF-2B having a higher affinity for eIF-2α-P than for eIF-2α (54). These results, coupled with the knowledge that the phosphate group of eIF-4E turns over more rapidly than does the polypeptide chain (53), suggest a model in which the association of eIF-4E with p220 is in dynamic equilibrium and is determined by eIF-4E phosphorylation.

As noted above, eIF-4F is isolated as a complex of three components: eIF-4A, eIF-4E, and p220. The fact that eIF-4A does not decrease under the same conditions which lead to a complete loss of eIF-4E and p220 (Fig. 9) suggests that the eIF-4E and p220 components may be more closely associated, physically, functionally, or from a regulatory standpoint. In fact, eIF-4A is present at a considerably higher level than is either eIF-4E or p220 (16). Also, some purification schemes yield a p220:eIF-4E complex with no eIF-4A (9, 22, 39).

Although there are several possibilities which might explain why a reduction of p220 is correlated with a loss in eIF-4E, the most likely explanation is that p220 is degraded by cellular proteases unless it is complexed with eIF-4E. This interpretation, as opposed to the hypothesis that eIF-4E affects p220 expression at the transcriptional or translational level, is supported by the nearly simultaneous loss of eIF-4E and p220 (Fig. 8). Such a mechanism could potentially reconcile the observations of Trachsel et al. (62), who found that the addition of purified eIF-4E was sufficient to stimulate translation in extracts of poliovirus-infected cells, with those of Grifo et al. (26), who found that eIF-4F rather than eIF-4E was required. Perhaps poliovirus infection initially causes an inactivation or sequestration of eIF-4E, and the failure of eIF-4E and p220 to interact leads to the degradation of p220. Thus, addition of eIF-4E alone could restore translation to cell extracts at early times during the course of viral infection but not later, when the p220 component is degraded.

From a practical standpoint, the AS cells provide a model system for the study of translation in mammalian cells and cell extracts which are deficient in eIF-4E and p220, akin to the yeast cell-free system containing a temperature-sensitive eIF-4E variant (2). It is yet to be determined whether other translational components are reduced in the AS cells, but the ability of highly purified eIF-4F to restore translation (Fig. 7A) together with the unchanged levels of eIF-4A (Fig. 9) argues against this. Previously, poliovirus-infected cells and their lysates, or cell-free translation systems derived from components of poliovirus-infected and normal cells, have been useful in investigating the role of p220 and eIF-4F (21, 46). An advantage of the AS cells and their extracts for this purpose is that the numerous biochemical and morphological changes occurring in poliovirus-infected cells (37) would not be complicating factors. The system will be especially suitable for studies of mRNA competition and discrimination both in vitro and in vivo.

ACKNOWLEDGMENTS

We are indebted to Riqiang Yan, University of Kentucky, for a purified preparation of eIF-4F, Diane Etchison, University of Kansas Medical Center, for antibodies to p220, and Hans Trachsel, University of Bern, Bern, Switzerland, for antibodies to eIF-4A.

This work was supported by grant GM20818 from the National Institute of General Medical Sciences, grant 3076 from the Council for Tobacco Research-USA, Inc., and by funds from the Office of the Vice Chancellor for Research and Graduate Studies, University of Kentucky Medical Center.

REFERENCES

1. **Altmann, M., C. Handschin, and H. Trachsel.** 1987. mRNA cap-binding protein: cloning of the gene encoding protein synthesis initiation factor eIF-4E from *Saccharomyces cerevisiae*. Mol. Cell. Biol. **7**:998–1003.
2. **Altmann, M., N. Sonenberg, and H. Trachsel.** 1989. Translation in *Saccharomyces cerevisiae*: initiation factor 4E-dependent cell-free system. Mol. Cell. Biol. **9**:4467–4472.
3. **Benne, R., and J. W. B. Hershey.** 1978. The mechanism of action of protein synthesis initiation factors from rabbit reticulocytes. J. Biol. Chem. **253**:3078–3087.
4. **Blum, S., M. Mueller, S. R. Schmid, P. Linder, and H. Trachsel.** 1989. Translation in *Saccharomyces cerevisiae*: initiation factor 4A-dependent cell-free system. Proc. Natl. Acad. Sci. USA **86**:6043–6046.
5. **Bonneau, A.-M., and N. Sonenberg.** 1987. Involvement of the 24-kDa cap-binding protein in regulation of protein synthesis in mitosis. J. Biol. Chem. **262**:11134–11139.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
7. **Brenner, C., N. Nakayama, M. Goebl, K. Tanaka, A. Toh-e, and K. Matsumoto.** 1988. *CDC33* encodes mRNA cap-binding protein eIF-4E of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **8**:3556–3559.
8. **Buckley, B., and E. Ehrenfeld.** 1986. Two-dimensional gel analyses of the 24-kDa cap binding protein from poliovirus-infected and uninfected HeLa cells. Virology **152**:497–501.
9. **Buckley, B., and E. Ehrenfeld.** 1987. The cap-binding protein complex in uninfected and poliovirus-infected HeLa cells. J. Biol. Chem. **262**:13599–13606.
10. **Contente, S., K. Kaylene, D. Rimoldi, and R. M. Friedman.** 1990. Expression of gene *rrg* is associated with reversion of NIH 3T3 transformed by LTR-c-H-*ras*. Science **249**:796–798.
11. **Darnbrough, C., S. Legon, T. Hunt, and R. J. Jackson.** 1973. Initiation of protein synthesis: evidence for messenger RNA-independent binding of methionyl-transfer RNA to the 40 S ribosomal subunit. J. Mol. Biol. **76**:379–403.
12. **De Benedetti, A., and R. E. Rhoads.** 1990. Overexpression of eukaryotic protein synthesis initiation factor 4E in HeLa cells results in aberrant growth and morphology. Proc. Natl. Acad. Sci. USA **87**:8212–8216.
13. **De Benedetti, A., and R. E. Rhoads.** 1991. A novel BK virus-based episomal vector for expression of foreign genes in mammalian cells. Nucleic Acids Res. **19**:1925–1931.
14. **Donahue, T. F., A. M. Cigan, E. K. Pabich, and B. Valavicius.**

1988. Mutations at a Zn(II) finger motif in the yeast eIF-2β gene alter ribosomal start-site selection during the scanning process. Cell **54**:621–632.

15. **Donaldson, R. W., C. H. Hagedorn, and S. Cohen.** 1991. Epidermal growth factor or okadaic acid stimulates phosphorylation of eukaryotic initiation factor 4F. J. Biol. Chem. **266**: 3162–3166.
16. **Duncan, R., S. C. Milburn, and J. W. B. Hershey.** 1987. Regulated phosphorylation and low abundance of HeLa cell initiation factor eIF-4F suggest a role in translational control. J. Biol. Chem. **262**:380–388.
17. **Edery, I., M. Hümbelin, A. Darveau, K. A. W. Lee, S. Milburn, J. W. B. Hershey, H. Trachsel, and N. Sonenberg.** 1983. Involvement of eucaryotic initiation factor 4A in the cap recognition process. J. Biol. Chem. **258**:11398–11403.
18. **Elferink, C. J., and J. P. Whitlock.** 1990. 2,3,7,8-Tetrachlorodibenzo-p-dioxin-inducible, Ah receptor-mediated bending of enhancer DNA. J. Biol. Chem. **265**:5718–5721.
19. **Etchison, D., and J. R. Etchison.** 1987. Monoclonal antibody-aided characterization of cellular p220 in uninfected and poliovirus-infected HeLa cells: subcellular distribution and identification of conformers. J. Virol. **61**:2702–2710.
20. **Etchison, D., and S. Fout.** 1985. Human rhinovirus 14 infection of HeLa cells results in the proteolytic cleavage of the p220 cap-binding complex subunit and inactivates globin mRNA translation in vitro. J. Virol. **54**:634–638.
21. **Etchison, D., J. Hansen, E. Ehrenfeld, I. Edery, N. Sonenberg, S. Milburn, and J. W. B. Hershey.** 1984. Demonstration in vitro that eucaryotic initiation factor 3 is active but that a cap-binding protein complex is inactive in poliovirus-infected HeLa cells. J. Virol. **51**:832–837.
22. **Etchison, D., and S. Milburn.** 1987. Separation of protein synthesis initiation factor eIF4A from a p220-associated cap binding complex activity. Mol. Cell. Biochem. **76**:15–25.
23. **Etchison, D., S. C. Milburn, I. Edery, S. Sonenberg, and J. W. B. Hershey.** 1982. Inhibition of HeLa cell protein synthesis following poliovirus infection correlates with the proteolysis of a 220,000-dalton polypeptide associated with eucaryotic initiation factor 3 and a cap binding protein complex. J. Biol. Chem. **257**:14806–14810.
24. **Green, P. J., O. Pines, and M. Inouye.** 1986. The role of antisense RNA in gene regulation. Annu. Rev. Biochem. **55**: 569–597.
25. **Griep, A. E., and H. Westphal.** 1988. Antisense *myc* sequences induce differentiation of F9 cells. Proc. Natl. Acad. Sci. USA **85**:6806–6810.
26. **Grifo, J. A., S. M. Tahara, M. A. Morgan, A. J. Shatkin, and W. C. Merrick.** 1983. New initiation factor activity required for globin mRNA translation. J. Biol. Chem. **258**:804–5810.
27. **Hansen, J., D. Etchison, J. W. B. Hershey, and E. Ehrenfeld.** 1982. Association of cap-binding protein with eucaryotic initiation factor 3 in initiation factor preparations from uninfected and poliovirus-infected HeLa cells. J. Virol. **42**:200–207.
28. **Hellmann, G. M., L.-Y. Chu, and R. E. Rhoads.** 1982. A polypeptide which reverses cap analogue inhibition of cell-free protein synthesis. Purification and binding to capped oligonucleotides. J. Biol. Chem. **257**:4056–4062.
29. **Hiremath, L. S., S. T. Hiremath, W. Rychlik, S. Joshi, L. L. Domier, and R. E. Rhoads.** 1989. In vitro synthesis, phosphorylation, and localization on 48S initiation complexes of human protein synthesis initiation factor 4E. J. Biol. Chem. **264**:1132–1138.
30. **Holt, J. T., T. V. Gopal, A. D. Moulton, and A. W. Nienhuis.** 1986. Inducible production of c-*fos* antisense RNA inhibits 3T3 cell proliferation. Proc. Natl. Acad. Sci. USA **83**:4794–4798.
31. **Izant, J. G., and H. Weintraub.** 1985. Constitutive and conditional suppression of exogenous and endogenous genes by anti-sense RNA. Science **299**:345–352.
32. **Jones, P. B. C., L. K. Durrin, D. R. Galeazzi, and J. P. Whitlock.** 1986. Control of cytochrome P_1-450 gene expression: analysis of a dioxin-responsive enhancer system. Proc. Natl. Acad. Sci. USA **83**:2802–2806.
33. **Joshi-Barve, S., W. Rychlik, and R. E. Rhoads.** 1990. Alteration of the major phosphorylation site of eukaryotic protein synthesis initiation factor 4E prevents its association with the 48 S initiation complex. J. Biol. Chem. **265**:2979–2983.
34. **Kaspar, R. L., W. Rychlik, M. W. White, R. E. Rhoads, and D. R. Morris.** 1990. Simultaneous cytoplasmic redistribution of ribosomal protein L32 mRNA and phosphorylation of eukaryotic initiation factor 4E after mitogenic stimulation of Swiss 3T3 cells. J. Biol. Chem. **265**:3619–3622.
35. **Kaufman, R. J., M. V. Davies, V. K. Pathak, and J. W. B. Hershey.** 1989. The phosphorylation state of eukaryotic initiation factor 2 alters translational efficiency of specific mRNAs. Mol. Cell. Biol. **9**:946–958.
36. **Khokha, R., P. Waterhouse, S. Yagel, P. K. Lala, C. M. Overall, G. Norton, and D. T. Denhardt.** 1989. Antisense RNA-induced reduction in murine TIMP levels confers oncogenicity on Swiss 3T3 cells. Science **243**:947–950.
37. **Koch, F., and G. Koch.** 1985. The molecular biology of polioviruses, p. 209, 226–266. Springer-Verlag, New York.
38. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
39. **Lamphear, B. J., and R. Panniers.** 1990. Cap binding protein complex that restores protein synthesis in heat-shocked Ehrlich cell lysates contains highly phosphorylated eIF-4E. J. Biol. Chem. **265**:5333–5336.
40. **Lazaris-Karatzas, A., K. S. Montine, and N. Sonenberg.** 1990. Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5′ cap. Nature (London) **345**:544–547.
41. **Manzella, J. M., W. Rychlik, R. E. Rhoads, J. W. B. Hershey, and P. J. Blackshear.** 1990. Insulin induction of ornithine decarboxylase. J. Biol. Chem. **266**:2383–2389.
42. **Marcus, A.** 1970. Tobacco mosaic virus ribonucleic acid-dependent amino acid incorporation in a wheat embryo system in vitro. Analysis of the rate-limiting reaction. J. Biol. Chem. **245**:955–961.
43. **McGarry, T. J., and S. Lindquist.** 1986. Inhibition of heat shock protein synthesis by heat-inducible antisense RNA. Proc. Natl. Acad. Sci. USA **83**:399–403.
44. **Melton, D. A.** 1985. Injected anti-sense RNAs specifically block messenger RNA translation in vivo. Proc. Natl. Acad. Sci. USA **82**:144–148.
45. **Milburn, S. C., J. W. Hershey, M. V. Davies, K. Kelleher, and R. J. Kaufman.** 1990. Cloning and expression of eukaryotic initiation factor 4B cDNA: sequence determination identifies a common RNA recognition motif. EMBO J. **9**:2783–2790.
46. **Morley, S. J., and J. W. B. Hershey.** 1990. A fractionated reticulocyte lysate retains high efficiency for protein synthesis. Biochemie **72**:259–264.
47. **Morley, S. J., and J. A. Traugh.** 1989. Phorbol esters stimulate phosphorylation of eukaryotic initiation factors 3, 4B, and 4F. J. Biol. Chem. **264**:2401–2404.
48. **Morley, S. J., and J. A. Traugh.** 1990. Differential stimulation of phosphorylation of initiation factors eIF-4F, eIF-4B, eIF-3 and ribosomal protein S6 by insulin and phorbol esters. J. Biol. Chem. **265**:10611–10616.
49. **Rhoads, R. E.** 1991. Initiation: mRNA and 60S subunit binding, p. 109–148. *In* H. Trachsel (ed.), Translation in eukaryotes. RC Press, Inc., Boca Raton, Fla.
50. **Rychlik, W., L. L. Domier, P. R. Gardner, G. M. Hellmann, and R. E. Rhoads.** 1987. Amino acid sequence of the mRNA cap-binding protein from human tissues. Proc. Natl. Acad. Sci. USA **84**:945–949.
51. **Rychlik, W., P. R. Gardner, T. C. Vanaman, and R. E. Rhoads.** 1986. Structural analysis of the messenger RNA cap-binding protein. Presence of phosphate, sulfhydryl, and disulfide groups. J. Biol. Chem. **261**:71–75.
52. **Rychlik, W., and R. E. Rhoads.** 1989. A computer program for choosing optimal oligonucleotides for filter hybridization, sequencing and in vitro amplification of DNA. Nucleic Acids Res. **17**:8543–8551.
53. **Rychlik, W., J. S. Rush, R. E. Rhoads, and C. J. Waechter.** 1990. Increased rate of phosphorylation-dephosphorylation of the translational initiation factor eIF-4E correlates with the

induction of protein and glycoprotein biosynthesis in activated B lymphocytes. J. Biol. Chem. **265**:19467–19471.
54. **Safer, B.** 1983. 2B or not 2B: regulation of the catalytic utilization of eIF-2. Cell **33**:7–8.
55. **Safer, B.** 1989. Nomenclature of initiation, elongation and termination factors for translation in eucaryotes. Recommendations 1988. Eur. J. Biochem. **186**:1–3.
56. **Safer, B., W. Kemper, and R. Jagus.** 1978. Identification of a 48 S preinitiation complex in reticulocyte lysate. J. Biol. Chem. **253**:3384–3386.
57. **Smit-McBride, Z., J. Schnier, R. J. Kaufman, and J. W. B. Hershey.** 1989. Protein synthesis initiation factor eIF-4D. Functional comparison of native and unhypusinated forms of the protein. J. Biol. Chem. **264**:18527–18530.
58. **Sonenberg, N.** 1988. Cap-binding proteins of eukaryotic messenger RNA: functions in initiation and control of translation. Prog. Nucleic Acid Res. Mol. Biol. **35**:173–207.
59. **Sonenberg, N., M. A. Morgan, W. C. Merrick, and A. J. Shatkin.** 1978. A polypeptide in eukaryotic initiation factors that crosslinks specifically to the 5′-terminal cap in mRNA. Proc. Natl. Acad. Sci. USA **75**:4843–4847.
60. **Tahara, S. M., M. A. Morgan, and A. J. Shatkin.** 1981. Two forms of purified m^7G-cap binding protein with different effects on capped mRNA translation in extracts of uninfected and poliovirus-infected HeLa cells. J. Biol. Chem. **256**:7691–7694.
61. **Trachsel, H., B. Erni, M. H. Schrier, and T. Staehelin.** 1977. Initiation of mammalian protein synthesis. II. The assembly of the initiation complex with purified initiation factors. J. Mol. Biol. **116**:755–767.
62. **Trachsel, H., N. Sonenberg, A. J. Shatkin, J. K. Rose, K. Leong, J. E. Bergmann, J. Gordon, and D. Baltimore.** 1980. Purification of a factor that restores translation of vesicular stomatitis virus mRNA in extracts from poliovirus-infected HeLa cells. Proc. Natl. Acad. Sci. USA **77**:770–774.
63. **Tuazon, P. T., S. J. Morley, T. E. Dever, W. C. Merrick, R. E. Rhoads, and J. A. Traugh.** 1990. Association of initiation factor eIF-4E in a cap binding protein complex (eIF-4F) is critical for and enhances phosphorylation by protein kinase C. J. Biol. Chem. **265**:10617–10621.
64. **Wang, A. M., M. V. Doyle, and D. F. Mark.** 1989. Quantitation of mRNA by the polymerase chain reaction. Proc. Natl. Acad. Sci. USA **86**:9717–9721.
65. **White, M. W., C. Degnin, J. Hill, and D. R. Morris.** 1990. Specific regulation by endogenous polyamines of translational initiation of S-adenosylmethionine decarboxylase mRNA in Swiss 3T3 fibroblasts. Biochem. J. **268**:657–660.
66. **Winston, S. E.** 1988. Western blotting, p. 10.8.1–10.8.6. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology, vol. 2. Green Publishing Associates and Wiley Interscience, New York.