Individual Overexpression of Five Subunits of Human Translation Initiation Factor eIF3 Promotes Malignant Transformation of Immortal Fibroblast Cells*‡

Received for publication, June 30, 2006, and in revised form, December 14, 2006 Published, JBC Papers in Press, December 14, 2006, DOI 10.1074/jbc.M606284200

Lili Zhang§, Xiaoyu Pan‡, and John W. B. Hershey‡1

From the §Department of Biochemistry and Molecular Medicine, School of Medicine, and the ‡Department of Molecular and Cell Biology, University of California, Davis, California 95616

Transcriptional and post-transcriptional regulatory mechanisms are commonly accepted paradigms of tumorigenesis. The view is emerging that deregulation of translation contributes importantly to cancer development, a role not generally appreciated before. Eukaryotic initiation factor eIF3 contains at least thirteen non-identical subunits, named from eIF3a to eIF3m, and plays an essential role in the rate-limiting initiation phase of translation. Increased mRNA and protein levels of the eIF3a, -3b, -3c, -3h, and -3i subunits have been detected in a wide variety of human tumors and are frequently identified as prognostic biomarkers for poor clinical outcome. However, it remains to be established whether up-regulation of eIF3 subunits is a consequence or a cause of the malignant phenotypes. Here we report that ectopic expression of eIF3a, -3b, -3c, -3h, or -3i in stably transfected NIH3T3 cells leads to a number of oncogenic properties: decreased doubling times, increased clonogenicity and viability, facilitated S-phase entry, attenuation of apoptosis, formation of transformed foci, and anchorage-independent growth. Only overexpression of the transforming subunits results in a stimulation of initiation and global protein synthesis rates and enhanced translation of poorly translated mRNAs that encode growth-regulating proteins, including cyclinD1, c-Myc, fibroblast growth factor-2, and ornithine decarboxylase, which may be responsible for oncogenic malignancy in the transformed cell lines. Based on these results, we hypothesize that eIF3 contributes to hyperactivation of the translation initiation machinery and thereby may play an important role in neoplasia. Cancer cells appear to acquire an aberrantly activated translational state to survive, suggesting that the initiation factors may be promising therapeutic targets for treating cancer.

Cancers develop when the expression of genes involved in cell proliferation is altered. Although most studies have focused on transcriptional control of gene expression, recent discoveries indicate that the regulation of protein synthesis also may be important in the etiology of cancer (1). Overexpression of the translation initiation factor eIF4E2 causes malignant transformation of immortal cells (2–7). eIF4E binds the m7G-cap structure at the 5′ terminus of mRNAs and plays a key role in the binding of mRNAs to ribosomes (8). Malignant transformation also follows the expression of a dominant negative mutant form of protein kinase R (9), a protein kinase that phosphorylates the α-subunit of eIF2 and inhibits protein synthesis by reducing the binding of the initiator methionyl-tRNA (8). Malignancy caused by reducing eIF2 inhibition also occurs by overexpression of a mutant form of eIF2 that cannot be phosphorylated by protein kinase R (10, 11). These observations have led to the hypothesis that failure to down-regulate protein synthesis results in a malignant phenotype.

Mechanisms leading to the regulation of protein synthesis most frequently affect the initiation phase of translation. Initiation involves the binding of initiator methionyl-tRNA and mRNA to the 40S ribosomal subunit, followed by junction with the 60S subunit to form an 80S initiation complex competent to enter the elongation phase of protein synthesis (8). The initiation pathway is promoted by at least 12 proteins called initiation factors (eIFs) that transiently interact with the components of protein synthesis. The largest of the eIFs is eIF3, a complex comprising 13 non-identical protein subunits in mammalian cells (8, 12–15). eIF3 binds to the solvent side of the 40S ribosomal subunit and interacts with a number of other initiation factors and mRNA, possibly playing an organizing role on the surface of the ribosome (16). Recent evidence indicates that eIF3 may be important in regulating the rate of initiation (14, 17–19). A detailed structure of human eIF3 is not available, although a cryoelectron microscopic reconstruction shows that its subunits are organized in a shape with five appendages (16). The functional roles of the individual subunits are not yet known, but five subunits (a, b, c, g, and i), which have homologs in yeast, may form a “core” complex to which the other subunits bind (20).

Aberrant mRNA and protein levels of several eIF3 subunits have been detected in a wide variety of solid tumors and cancer cell lines. Different isoforms of eIF3a are overexpressed in mouse melanoma and HeLa cells (21), human breast (22), cervical (23), esophageal (24), lung (21), and gastric cancers (24). eIF3b is overexpressed in human breast carcinoma (25). Ele-
Blotting
trol cells following the manufacturer's instructions (Flp-In sys-
strumentation to express individually each of twelve eIF3 subunits
malignant phenotype. This unexpected result suggests (but does not prove) that eIF3 may play a causal role in neoplasia, consistent with misregulation of translation being a contributory factor to cancer pathology. eIF3 subunit overexpression not only results in a modest increase in the global rate of protein synthesis but also selectively enhances translation of several malignancy-associated mRNAs, which may be responsible for the oncogenic properties of the transformed cells.

**PROTEIN EXTRACTION, IMMUNOPRECIPITATION, AND WESTERN BLOTTING**

**Cells and Plasmid Constructs**—cDNAs encoding full-length eIF3 subunits were altered to provide a unique Ndel site at the initiator AUG: eIF3a (D50929), eIF3b (U78525), eIF3c (U46025), eIF3d (U54558), eIF3e (U54562), eIF3f (U94855), eIF3g (U96074), eIF3h (U54559), eIF3i (U39067), eIF3j (U97670), eIF3k (NM013234) and eIF3l (BC029265); eIF3m was not yet identified when this work began, so it was not incorporated into the study. Each cDNA was inserted into the Ndel-Xhol sites of pAMV-pA-HA (37), which was engineered to include a hemagglutinin (HA) tag downstream of a HindIII site followed by an in-frame ATG within the Ndel site. The resulting HA-tagged sequences were moved into pcDNA5/FRT to build pcDNA5/eIF3x expression vectors (Invitrogen), and the correctness of constructs was confirmed by DNA sequencing. NIH3T3 cells from the American Type Culture Collection were grown in Dulbecco's modified Eagle's medium supplemented with NIH3T3 cells from the American Type Culture Collection were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. All cell culture materials were obtained from Mediatech Inc. We constructed the twelve Flp-in-3T3-eIF3x expression vectors and Flp-in-3T3- pcDNA5 control vectors following the manufacturer's instructions (Flp-In system, Invitrogen).

**Analysis of Specific mRNAs in Polysome Fractions**—3T3-eIF3x cells were lysed and subjected to centrifugation on 10–30% sucrose gradients containing 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM MgCl2, and 1 mM dithiothreitol. Gradients were centrifuged in a Beckman SW41 rotor at 40,000 rpm for 90 min, scanned at A254 and collected into nine fractions. *Escherichia coli* rRNA (1 μg, Roche Applied Science) was added to each fraction, and total RNA was extracted with TRIzol reagent (Invitrogen) and analyzed with an Applied Biosystems 7000 sequence detector, and the value for each gradient fraction is reported as the percent of the total amount in all nine fractions.

**Statistical Analyses**—Statistical significance of the data presented as the mean ± S.E. was analyzed by Student's *t* test. A *p* value below 0.05 was considered to be significant.

**RESULTS**

**Overexpression of Twelve eIF3 Subunits in a Flp-In-3T3-eIF3x Expression System**—To determine whether or not overexpression of individual eIF3 subunits leads to cellular transformation, we constructed twelve Flp-In-3T3-eIF3x expression cell lines (for subunits a–l) and one Flp-in-3T3-pcDNA5 control cell line (v) (supplemental Fig. S1) following the manufacturer's instructions. Briefly, we stably transfected pFRT/IacZeo into early-passage immortal NIH3T3 fibroblast cells to generate cells containing an integrated Flp-recombination target. A cell clone that expresses β-galactosidase at a high level was chosen as the Flp-In-3T3 host cell line. Subsequently, we cotransfected the host cells with a pcDNA5/eIF3x expression vector containing an HA-tagged eIF3x cassette and pOG44. The Flp-recombinase expressed from pOG44 catalyzes a site-specific homologous recombination between the Flp-recombination target sites in the host cell genome and in the pcDNA5/eIF3x

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Plasmid Constructs**—cDNAs encoding full-length eIF3 subunits were altered to provide a unique Ndel site at the initiator AUG: eIF3a (D50929), eIF3b (U78525), eIF3c (U46025), eIF3d (U54558), eIF3e (U54562), eIF3f (U94855), eIF3g (U96074), eIF3h (U54559), eIF3i (U39067), eIF3j (U97670), eIF3k (NM013234) and eIF3l (BC029265); eIF3m was not yet identified when this work began, so it was not incorporated into the study. Each cDNA was inserted into the Ndel-Xhol sites of pAMV-pA-HA (37), which was engineered to include a hemagglutinin (HA) tag downstream of a HindIII site followed by an in-frame ATG within the Ndel site. The resulting HA-tagged sequences were moved into pcDNA5/FRT to build pcDNA5/eIF3x expression vectors (Invitrogen), and the correctness of constructs was confirmed by DNA sequencing. NIH3T3 cells from the American Type Culture Collection were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. All cell culture materials were obtained from Mediatech Inc. We constructed the twelve Flp-in-3T3-eIF3x expression vectors and Flp-in-3T3-pcDNA5 control vectors following the manufacturer’s instructions (Flp-In system, Invitrogen).

**Protein Extraction, Immunoprecipitation, and Western Blotting**—Cells (1 × 106) were lysed in NIH3T3 cell lysis buffer (ProSci Inc., Poway, CA). Immunoprecipitates were prepared by incubating the lysates either with anti-HA-agarose resin (Sigma) or with monoclonal anti-eIF3a (gift of J. T. Parsons, University of Virginia) and Dynabeads protein-A (Dynal Biotech). The following primary antibodies were used in immunoblotting: monoclonal anti-HA and anti-β-actin (Sigma); polyclonal anti-eIF2α and monoclonal anti-phospho-eIF2α (Cell Signaling Technology, Danvers, MA); polyclonal anti-eIF3 (38); polyclonal anti-eIF4G, anti-eIF4B, anti-eIF4E (Cell Signaling Technology) and anti-4EBP1 (Abcam, Cambridge, UK); monoclonal anti-eIF3a and anti-eIF3c (gift from D. R. Scoles, UCLA) and anti-eIF3j (gift from C. S. Fraser, UC Berkeley); polyclonal anti-eIF3b, anti-eIF3e, anti-eIF3f, and anti-eIF3h (Santa Cruz Biotechnology, Santa Cruz, CA); and polyclonal anti-PARP (Cell Signaling Technology). The blots were incubated with alkaline phosphatase-conjugated secondary antibodies (Sigma), developed in 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium solution (Sigma), scanned with a photo-scanner, and analyzed with AlphaEaseFC Image software.

**Analysis of Specific mRNAs in Polysome Fractions**—To determine whether or not overexpression of individual eIF3 subunits leads to cellular transformation, we constructed twelve Flp-In-3T3-eIF3x expression cell lines (for subunits a–l) and one Flp-in-3T3-pcDNA5 control cell line (v) (supplemental Fig. S1) following the manufacturer’s instructions. Briefly, we stably transfected pFRT/IacZeo into early-passage immortal NIH3T3 fibroblast cells to generate cells containing an integrated Flp-recombination target. A cell clone that expresses β-galactosidase at a high level was chosen as the Flp-In-3T3 host cell line. Subsequently, we cotransfected the host cells with a pcDNA5/eIF3x expression vector containing an HA-tagged eIF3x cassette and pOG44. The Flp-recombinase expressed from pOG44 catalyzes a site-specific homologous recombination between the Flp-recombination target sites in the host cell genome and in the pcDNA5/eIF3x

**REFERENCES**
Oncogenic Role of Five Human eIF3 Subunits

Individual overexpression of twelve eIF3 subunits in a Flp-In-T2 cells was achieved by transfecting each cell line with a vector expressing a particular eIF3 subunit. The Western blots (WB) were probed with anti-HA antibodies to detect the full-length HA-eIF3x proteins. The bar graph (B) shows the relative levels of HA-eIF3x in the different cell lines, with the y-axis representing the level of expression. The x-axis represents the cell lines, and the bars indicate the amount of HA-eIF3x detected in each line.

To demonstrate that the expression constructs produce the predicted protein products, we treated each cell lysate with anti-HA-agarose and analyzed the HA-eIF3x proteins by Western blotting with anti-HA. A single major band corresponding to the predicted protein products was detected in each eIF3x immunoprecipitate, whereas eIF3a was too weak to be detected, although a low level is seen when the cell immunoprecipitate is examined (Fig. 1A). The different levels of accumulation of the HA-eIF3x proteins are likely due to variable degradation or insolubility, especially because each overexpressed subunit likely exists in large part as a free protein outside the eIF3 complex. Similar instability/insolubility is seen when eIF3a is expressed in recombinant baculovirus-infected S9 insect cells (37) or when eIF3i is overexpressed in NIH3T3 cells (27, 28).

Ectopic Expression of eIF3a, eIF3b, eIF3c, eIF3h, or eIF3i Stimulates Cell Growth and Induces Neoplastic Transformation—The growth characteristics of the twelve eIF3x cell lines were compared with those of the control 3T3-pcDNA5 cells. As shown in Fig. 2A, the growth curves can be grouped into three categories: the fast growing cells (a, b, c, h, and i) have doubling times of 19.2–21.8 h; normal growing cells (d, g, j, and k) have doubling times of 23.1–25.3 h comparable to 26.4 h for the control 3T3-pcDNA5 cells; and slow growing cells (e and f) have doubling times of 31.0–31.8 h. Although 3T3-pcDNA5 cells become confluent on day 5 after plating, saturation densities (the plateau values shown in Fig. 2A) of the fast growing cells are 1.7–1.9-fold greater than those of the normal growing cells, whereas those of the slow growing cells are 1.5-fold lower. Thus, the fast growing cells grow faster in exponential phase and reach higher saturation densities.

The fast growing cells also exhibit superior colony-forming ability when plated at very low density. As shown in supplemental Fig. S2, the colony density of cells expressing the a, b, c, h, or i subunit is much greater than the control cells, whose colonies resemble those from cells expressing the g or j subunits. The d, k, and l subunits display slightly greater clonogenicity than the control cells but not nearly as great as the a, b, c, h, and i group. The slow growing cells expressing the e or f subunits show even lower colony densities. Colony densities were quantitated and are reported in Fig. 2B. A second method to assess cell proliferation is to use the MTT assay to measure the mitochondrial dehydrogenase activity indicative of metabolically active cells. The MTT assay results are plotted in Fig. 2B and closely resemble those from the colony formation assay. Lastly, cell proliferation rates in the eIF3x cell lines were assessed by determining the percentage of cells residing in the S-phase and undergoing dynamic DNA synthesis (bromodeoxyuridine-positive) by using flow cytometry (supplemental Fig. S3). We found a larger proportion (49–56%) of the fast growing cells to be bromodeoxyuridine-positive and reside in S-phase, compared with 36–42% of the normal growing cells and 33–34% of the slow growing cells (Fig. 2C). Therefore, the fast growing cells expressing the a, b, c, h, or i subunit proliferate

vector. Therefore, each of the eIF3 subunit cDNAs is integrated into the same locus in the chromosome, allowing comparative analysis of the effects of their expression.

To demonstrate that the expression constructs produce the predicted protein products, we treated each cell lysate with anti-HA-agarose and analyzed the HA-eIF3x immunoprecipitates by immunoblotting with anti-HA. A single major band was detected in each eIF3x immunoprecipitate, whereas 3T3-pcDNA5 cells exhibited no anti-HA band (Fig. 1A). For each subunit, the apparent molecular weight as determined by migration in SDS-polyacrylamide gels was identical to that of the corresponding subunit derived from eIF3 purified from HeLa cells (data not shown), indicating that full-length subunits are being produced.

The extent of subunit overexpression relative to endogenous eIF3 levels was calculated from anti-HA immunoblots of eIF3x lysates (Fig. 1, B lower panel) and C as described in the figure legend. The b, c, f, g, j, and k subunits accumulate to ~4-fold or greater above the endogenous eIF3 level, the d, e, h, and l subunits are 2.5- to 3.0-fold higher, whereas eIF3i is only ~1.5-fold higher (Fig. 1B, top panel). The immunoblot signal for HA-eIF3a is too weak to be detected, although a low level is seen when the cell immunoprecipitate is examined (Fig. 1A). The different levels of accumulation of the HA-eIF3x proteins are likely due to variable degradation or insolubility, especially because each overexpressed subunit likely exists in large part as a free protein outside the eIF3 complex. Similar instability/insolubility is seen when eIF3a is expressed in recombinant baculovirus-infected S9 insect cells (37) or when eIF3i is overexpressed in NIH3T3 cells (27, 28).
faster, are more active metabolically, and form denser colonies than normal cells. These data also suggest that these cells may have been malignantly transformed.

Loss of contact inhibition and loss of anchorage-dependent growth are hallmarks of malignant cells and are considered to be two of the general phenotypes of an oncogene. We assayed foci formation using the fifth passage of pooled clones from eIF3x cells. The fast growing cells (columns a, b, c, h, and i) give rise to foci 2–3 weeks after inoculation (Fig. 3A, row 1), whereas

FIGURE 2. Stimulation of cell proliferation in the transformed cells. A growth curves were obtained by seeding cells onto 35-mm plates at a density of $1 \times 10^5$ cells/plate containing Dulbecco’s modified Eagle’s medium (Mediatech, Inc.) supplemented with 10% fetal calf serum and counting every 24 h with a hemocytometer. The cells separate into three groups: fast growing cells (a, b, c, h, and i), normal growing cells (d, g, j, k, l, and v), and slow growing cells (e and f). The values shown are the average of three independent experiments each done in triplicate.

B, quantitation of clonogenicity and MTT assay. Colony formation units (shown by the gray bars) were measured by seeding 10^5 cells onto 10-cm plates, incubating for 2–3 weeks, and quantified relative to the 3T3pcDNA5 control cells (normalized to 1). The tetrazolium salt-based cell viability assay (MTT) (black bars) was performed by using Cell Proliferation kit I (Roche Applied Science). Values shown are mean ± S.D. of three independent experiments done in triplicate. **, p < 0.01; *, p < 0.05.

C, quantitation of bromodeoxyuridine-positive/S-phase cells by flow cytometry. Cells were grown in Dulbecco’s modified Eagle’s medium and analyzed with an FITC-bromodeoxyuridine flow kit (BD Pharmingen) and BD FACScan and CellQuest software, as shown in supplemental Fig. 53. The values shown are mean ± S.D. of three independent experiments done in triplicate. ***, p < 0.01; **, p < 0.05; as in B.

FIGURE 3. Overexpression of eIF3a, eIF3b, eIF3c, eIF3h, or eIF3i induces oncogenic transformation. A, abnormal growth properties of the transformed cells. Each column represents a specific cell line, as labeled at the top (e.g. a = 3T3-eIF3a cells). Row 1: the fifth passage of 3T3-eIF3x cells (a–l) and the vector control (v) were overgrown for 3 weeks to develop transformed foci by placing $5 \times 10^4$ cells in 0.3% (w/v) agar containing normal media, and overlaying them with solidified 0.6% (w/v) agar in 35-mm plates. The cells were fixed, stained by 0.5% methylene blue, and photographed. The intensely stained colonies are transformed foci. Row 2: transformed foci formed by 10th passage 3T3-eIF3x cells analyzed as in row 1. Row 3: 400× magnification of row 2 to show the characteristic crisscross and stacked growth of foci cells. Row 4: 400× magnification of a region of the cells from row 2 that show, from left to right, loss of contact inhibition, disoriented organization, spindle-shaped and multilayered growth, and contact inhibition of the transformed cells, and monolayer growth of the vector control (v). Row 5: typical colonies recovered from soft agar after 3 week incubation of the above cell lines. B, quantitation of soft agar colony assays. The colonies in row 1 above were counted at day 21 after plating, and colony formation efficiency (gray bars) was calculated as the number of colonies divided by the number of cells plated × 100. The colony size was measured and is plotted as the percentage of big colonies (>0.5 cm) in total colonies and shown as black bars. Values shown are mean ± S.D. of three independent experiments done in triplicate. ***, p < 0.01; as in Fig. 2B.

Loss of contact inhibition and loss of anchorage-dependent growth are hallmarks of malignant cells and are considered to be two of the general phenotypes of an oncogene. We assayed foci formation using the fifth passage of pooled clones from eIF3x cells. The fast growing cells (columns a, b, c, h, and i) give rise to foci 2–3 weeks after inoculation (Fig. 3A, row 1), whereas
Oncogenic Role of Five Human eIF3 Subunits

no foci are observed at 3 weeks for the control 3T3-pcDNA5 cells (column v) nor for the normal and slow growing cells (results not shown). The number of foci in the fast growing cells is ~20-fold higher when tested at passage 10, indicating the selective advantage of transformed cells (Fig. 3A, row 2). Microscopical examination of the foci reveals multilayered growth and a disoriented morphology (Fig. 3A, row 3). The malignant transformants are highly refractile, have spindle-like projections, and displayed reduced contact inhibition, resulting in a piling up of cells (Fig. 3A, row 4) with a 2- to 2.5-fold increase in saturation density as compared with the vector control. In contrast, 3T3-pcDNA5 control cells grow in a monolayer, show contact inhibition and morphologically resemble normal fibroblast cells (Fig. 3A, vector v column). All of the fast growing cells show the anchorage-independent phenotype and grow in 0.3% agar (Fig. 3A, row 5), whereas the other cell lines do not (column v and results not shown). The colony formation efficiency of the fast growing cells is 40- to 50-fold higher than the normal and slow growing cells, as quantitated in Fig. 3B. Immunoblot analysis of protein extracts derived from the soft agar colonies of the fast growing cells reveal high levels of the corresponding eIF3 subunits (supplemental Fig. S4), confirming that the colonies comprise overproducing cells. The results from both the foci and soft agar assays demonstrate that overexpression of either the a, b, c, h, or i subunits of eIF3 is associated with malignant transformation of cultured immortal cells.

Transformed Cell Lines Resist Apoptosis and Show a Reduced Growth Factor Requirement—Programmed cell death (apoptosis) also is closely affiliated with the rate of protein synthesis, and many cancerous cells are resistant to induction of apoptosis. To assess this characteristic in the cell lines that overexpress eIF3x, we treated cells with camptothecin to induce apoptosis. Apoptosis was measured by annexin-V-FITC and propidium iodide (PI) double staining. Annexin-V is a Ca\(^{2+}\)-dependent phospholipid-binding protein and has a high affinity for membrane phosphatidyserine, which is exposed to the outer leaflet of the plasma membrane during apoptosis. Thus, viable cells are annexin-V-FITC- and PI-negative, early apoptotic cells are annexin-V-FITC-positive and PI-negative, and late apoptotic and dead cells are double stained. The normal growing cells (d, g, j, k, l, and v) and slow growing cells (e and f) display dynamic migration to the apoptotic window at 8 h after induction, whereas the transformed cells (a, b, c, h, and i) show strong resistance (Fig. 4A). The transformed cells exhibit ~6- to 7-fold fewer apoptotic cells than the normal and slow growing cells (Fig. 4B).

A second approach to assess caspase-3-mediated apoptosis is to measure proteolytic cleavage of the 116-kDa poly(ADP-ribose) polymerase (PARP) to an 85-kDa digestion fragment. The 85-kDa PARP cleavage product is detected in all of the normal and slow growing cells at 8 h after induction, whereas the transformed cells did not show appreciable proteolysis of PARP at the same time point (Fig. 4C). Morphological changes also were examined under the light microscope at 4, 8, and 12 h after induction (Fig. 4D, row LM). At 4 h, the control cells (v) begin to shrink and undergo chromatin aggregation to form cap-like regions under the nuclear membrane; at 8 h, they show cytoplasmic blebs characteristic of apoptotic cells; and at 12 h, most cells are rounded or fragmented into smaller bodies. However, the resistant cells (eIF3h) are able to maintain normal NIH3T3 morphology at 12 h after induction. 4',6-Diamidino-2-phenylindole staining and fluorescence microscope inspection show chromatin condensation in sensitive control cells, and distributed chromosome in the resistant eIF3h cells at the various times (Fig. 4D, row FM).

To assess the growth dependence on serum of the cell lines overproducing eIF3x, cells were grown in medium containing 0.1% serum to induce apoptosis. Under conditions of serum depletion, the normal and slow growing cells rapidly undergo growth arrest, but the transformed cells (a, b, c, h, and i) continue to proliferate (Fig. 4E). Morphological changes indicative of apoptosis were observed in the normal and slow growing cells after 48 h (data not shown). We conclude that the malignant transformed cells have a reduced requirement for serum growth factors and resist entry into apoptosis.

How Do the Five Overexpressed eIF3 Subunits Cause Malignant Transformation?—The finding that overexpression of any of 5 of the 12 eIF3 subunits of eIF3 results in transformation is surprising and suggests that regulation of protein synthesis through eIF3 is critical for growth control. To better understand how such regulation occurs, we first asked whether or not each of the overexpressed HA-tagged subunits is incorporated into the endogenous eIF3 complex. 3T3-eIF3x cell lysates were subjected to immuno precipitation with anti-HA resin, followed by SDS-PAGE, and immunoblotting analysis of the precipitated proteins with anti-eIF3 antiserum. As shown in Fig. 5A, the anti-HA antibody brings down other eIF3 subunits in the cell lines expressing the b, c, f, g, h, and i subunits, indicating that these subunits are incorporated into the eIF3 complex. Identification of the largest eIF3 subunits in these immunoprecipitates is confirmed by immunoblotting with specific antibodies (Fig. 5B). However, failure to immunoprecipitate eIF3 with anti-HA resin does not necessarily mean that a HA-tagged subunit is not a part of eIF3, because incorporation may result in the masking of the HA epitope. An alternate approach to the problem is to immunoprecipitate eIF3 with a highly specific antibody, then test for the presence of the HA-tagged subunit. As shown in Fig. 5C, treatment of 3T3-eIF3x cell lysates with a monoclonal anti-3a antibody causes 10 of the 12 HA-tagged subunits to precipitate; only HA-tagged eIF3a and eIF3h are not detected, the former likely due to its low expression. We conclude that nearly all overexpressed subunits are capable of incorporating into the endogenous eIF3 complex. Consistent with this finding, we also detect the HA-eIF3 subunits in the 40S and polysome regions of polysome profiles generated by sucrose gradient centrifugation analysis of 3T3-eIF3x lysates (supplemental Fig. S5). Their apparent interaction with ribosomes likely is due to their incorporation into eIF3, because the individual subunits of eIF3 (except for eIF3j) do not bind stably to ribosomes (37).

To shed further light on how the HA-tagged eIF3 subunits function in the malignantly transformed cells, we determined if they are located in the cytoplasm or the nucleus. HA-eIF3x cells were treated with anti-HA coupled to Alexa Fluor-488 (green), and used 7-ADD staining (red) to identify nuclei. The parental 3T3 cells and 3T3-pcDNA5 cells generate no green fluores-
FIGURE 4. Overexpression of eIF3a, -b, -c, -h, or -i impedes induction of apoptosis. A, detection of apoptosis by annexin-V-FITC and propidium iodide (PI) staining. Cells were treated with 10 μM camptothecin for 12 h and analyzed with the annexin-V-FITC apoptosis detection kit I (BD Pharmingen) by using BD FACScan and CellQuest software. Normal cells are not stained and appear in the lower left window; early apoptotic cells are stained by annexin-V-FITC and appear in the lower right window; late apoptotic and dead cells are double-stained by annexin-V-FITC and PI, and appear in the upper right window. Normal growing cells (d, g, j, k, l, and v) and slow growing cells (e and f) display dynamic migration to the apoptotic window at 8 h after induction, whereas transformed cells (a, b, c, h, and i) display resistance. B, quantitation of membrane exclusion assay shown in panel A. Values shown are mean ± S.D. of three independent experiments done in triplicate. **, p < 0.01; *, p < 0.05; as in Fig. 2B. C, caspase activity was detected by immunoblotting with monoclonal anti-poly-(ADP-ribose)-polymerase (Sigma). No 85-kDa caspase-digested product is detected in the transformed cells (a, b, c, h, and i) but is seen with the control and other eIF3x cells. The Western blot with anti-actin below is used as a gel loading control. D, morphological examination of apoptosis by light (LM) and fluorescence (FM) microscopy (400× magnification). In the LM and FM rows, the left-most panel shows eIF3h cells 12 h after induction with camptothecin; the three panels further to the right show 3T3-pcDNA5 control (v) cells at 4, 8, and 12 h after induction, exhibiting chromatin aggregation, cytoplasmic blebs, and fragmentation. In the FM row, cell nuclei were stained with 4',6-diamidino-2-phenylindole (Sigma); the eIF3h cells exhibit distributed chromosomes, whereas control v cells reveal different extents of chromatin aggregation and micronuclei formation at corresponding time points. E, the eIF3x cell lines were grown for 12 h on plates in Dulbecco’s modified Eagle’s medium supplemented with 0.1% serum and counted, as described in the legend to Fig. 2A. **, p < 0.01; as in Fig. 2B.
cence signal with anti-HA, nor did nonspecific IgG1 when substituted for anti-HA (supplemental Fig. S6). Most of the HA-tagged subunits (including all of the transforming subunits a, b, c, h, and i) are present exclusively in the cytoplasm, whereas the e, f, and k subunits localize predominately in the nuclear compartment. Immunofluorescence analysis of 3T3-eIF3x cell lines with anti-eIF3 serum confirms the view that eIF3 is a cytoplasmic protein, consistent with its role in protein synthesis (supplemental Fig. S6).

**Elevated Levels of the eIF3 Complex May Be Responsible for Enhanced Translation Rates and Malignant Transformation**—We next focused our attention on the effect of overproducing a single eIF3 subunit on the level of the entire eIF3 complex in the transformed cells. Equal amounts of protein from 3T3-eIF3x cell lysates were subjected to SDS-PAGE and immunoblotting with highly specific anti-eIF3a, anti-eIF3b, anti-eIF3f, anti-eIF3h, and anti-eIF3j to determine the level of eIF3 in these cells. Surprisingly, only the transformed cells expressing the a, b, or c subunit show clearly higher eIF3a, eIF3b, eIF3f, eIF3h, and eIF3j levels, whereas the non-transformed cell lines and the 3T3-eIF3h and 3T3-eIF3i cell lines exhibit very modest or no enhancement (Fig. 6, A and B). Equal loading of the immunoblotted gels was confirmed by blotting with an anti-actin antibody and is confirmed by the constant levels of other proteins among the panels (Supplemental Fig. S6).

**FIGURE 5.** Overexpressed HA-eIF3x proteins are incorporated into the endogenous eIF3 complex. A, 3T3-eIF3x cells were lysed and subjected to immunoprecipitation with anti-HA-resin as described under “Experimental Procedures.” The immunoprecipitations were analyzed by SDS-PAGE and immunoblotting with anti-eIF3 antisera. The panel shows a photograph of the developed blot. Bands corresponding to the a, b/c, f, and j subunits of eIF3 are labeled on the right. B, the same immunoprecipitations from panel A were analyzed similarly, but the blots were developed with anti-eIF3a, anti-eIF3b, and anti-eIF3c to confirm the presence of these subunits in the IPs. Only a portion of the blots is shown. C, the same lysates as in A and B were subjected to immunoprecipitation with the highly specific monoclonal anti-eIF3a antibody, followed by immunoblotting with anti-HA to detect the presence of the overexpressed subunits. All HA-eIF3x proteins (except a and h) appear in the precipitated endogenous eIF3 complex. The arrow on the left side of each lane identifies the immunoreactive band representing the full-length HA-eIF3x as deduced from its expected migration distance.

**FIGURE 6.** Overexpression of eIF3a, eIF3b, or eIF3c increases the level of the endogenous eIF3 complex. A, the 3T3-eIF3x (a–l) and control (v) cells were lysed, and equal amounts of protein were evaluated by SDS-PAGE and immunoblotting with anti-eIF3a, anti-eIF3b, anti-eIF3f, anti-eIF3h, and anti-eIF3j. Equal loading of the lanes was confirmed by blotting with anti-actin. The 3T3-eIF3x, 3T3-eIF3b, and 3T3-eIF3c cell lines show greater levels of eIF3 complex (represented by intensified a, b, f, h, and j bands). The lysates were also analyzed for eIF4G, eIF4B, eIF4E, eIF4EBP1, eIF2a, and phospho-eIF2a, where the band intensities did not vary between cell lines. B, quantitation of eIF3a, eIF3b, eIF3f, eIF3h, and eIF3j by optical density measurement in the indicated cell lines is shown in columns. A 40–50% -fold increase of eIF3 is observed in the 3T3-eIF3a, 3T3-eIF3b, and 3T3-eIF3c cell lines. Values shown are mean ± S.D. of two independent experiments. **, p < 0.01; as in Fig. 2B.
Oncogenic Role of Five Human eIF3 Subunits

Initiation factors. The enhanced levels of the a, b, f, h, and j subunits in the 3T3-eIF3a, 3T3-eIF3b, and 3T3-eIF3c cell lines suggest that the eIF3 complex is elevated in these cells. How overexpression of a single eIF3 subunit might alter the level of eIF3 or other eIF3 subunits is not obvious; this unexpected result is discussed below. The elevated level of eIF3 in the 3T3-eIF3a, 3T3-eIF3b, and 3T3-eIF3c cell lines may result in higher eIF3 activity and thus be responsible for the enhanced rate of protein synthesis in these cells, although this conjecture has not been established rigorously. Unfortunately, no method exists to measure the activity of eIF3 in vivo or in crude lysates.

A possible explanation for the higher levels of the a, b, c, f, and h subunits is that many or all translation components are enhanced to support the faster growth of the malignant cells. To assess this possibility, we subjected the same cell lysates to immunoblot analysis for eIF4G, eIF4A, eIF4E, eIF4EIP1, eIF2a, and phosphorylated eIF2α levels. The band intensities for these proteins do not vary but are constant for all 3T3-eIF3x cell lines (Fig. 6A). This suggests that eIF3x overproduction does not result in a significant increase in the levels of the general translational apparatus, but rather has a more limited effect on eIF3 in the case of eIF3a, eIF3b, and eIF3c overexpression.

Does the overexpression of an eIF3 subunit affect the rate of in vivo protein synthesis? We pulse-labeled 3T3-eIF3x cells with [35S]methionine to measured the rate of global protein synthesis. The protein synthesis rates of the transformed cell lines are between 35 and 49% higher than that of the normal growing cells (only up to a 16% increase), whereas the slow growing cells are lower by 20% to 26% (Fig. 7A). Thus, overexpression of only subunits a, b, c, h, or i results in significant activation of protein synthesis. To confirm this conclusion and determine if the effect is at the level of initiation, polysome profiles for the 12 3T3-eIF3x cell lines and the 3T3-pcDNA5 control were obtained (Fig. 7B). Heavy polysomes are seen in all of the cell lines except those where eIF3e and eIF3f are overexpressed. A shift of ribosomes toward the heaviest polysomes, relative to the control cell line, is seen most clearly in the cell lines overexpressing the b, c, and h subunits (see polysome to monosome ratios reported in Fig. 7A). A more modest shift is seen in the a and i cell lines, and even less so in the g, k, and l cell lines. The presence of larger polysomes indicates that eIF3x overproduction does not result in a significant increase in the levels of the general translational apparatus, but rather has a more limited effect on eIF3 in the case of eIF3a, eIF3b, and eIF3c overexpression.
growth factor-2, and ornithine decarboxylase mRNAs in transformed (c and h) and non-transformed (g and v) cells. Cell lysates were fractionated by sucrose gradient centrifugation (Fig. 8A), and mRNAs were purified from the gradient fractions and analyzed by TaqMan real-time reverse transcription-PCR as described under “Experimental Procedures.” A, schematic representation of the polysomes obtained by sucrose gradient centrifugation. Nine fractions (numbered below the figure) were collected for analysis, with fraction 1 representing the top of the gradient. The sedimentation positions of 40 S, 60 S, and 80 S ribosomes and 5-mer polysomes (messages containing five ribosomes) are labeled. RNA was extracted from individual fractions and analyzed by TaqMan real-time reverse transcription-PCR using specific primers and probes. B–F, quantitation of mRNA in each gradient fraction is reported as the percentage of the total amount present in all nine fractions. Polysomal distributions of B (c-Myc), C (cyclinD1), D (fibroblast growth factor 2), E (ornithine decarboxylase, ODC), and F (glyceraldehyde-3-phosphate dehydrogenase, GAPDH) mRNAs in extracts prepared from transformed (c and h) and non-transformed (g and v) cells are shown. The values are representative of two independent experiments done in triplicate.

FIGURE 8. Overexpression of eIF3c or eIF3h specifically stimulates the translation of mRNAs involved in cell proliferation. 3T3-eIF3c, 3T3-eIF3g, 3T3-eIF3h, and 3T3-pcDNA5 control cells (3 × 10⁶ cells each) were analyzed by sucrose gradient centrifugation, and specific mRNA levels in the gradient fractions were measured by real-time reverse transcription-PCR as described under “Experimental Procedures.” A, schematic representation of the polysomes obtained by sucrose gradient centrifugation. Nine fractions (numbered below the figure) were collected for analysis, with fraction 1 representing the top of the gradient. The sedimentation positions of 40 S, 60 S, and 80 S ribosomes and 5-mer polysomes (messages containing five ribosomes) are labeled. RNA was extracted from individual fractions and analyzed by TaqMan real-time reverse transcription-PCR using specific primers and probes. B–F, quantitation of mRNA in each gradient fraction is reported as the percentage of the total amount present in all nine fractions. Polysomal distributions of B (c-Myc), C (cyclinD1), D (fibroblast growth factor 2), E (ornithine decarboxylase, ODC), and F (glyceraldehyde-3-phosphate dehydrogenase, GAPDH) mRNAs in extracts prepared from transformed (c and h) and non-transformed (g and v) cells are shown. The values are representative of two independent experiments done in triplicate.

DISCUSSION

The finding that overexpression of any one of five different eIF3 subunits leads to malignant transformation of immortal NIH3T3 fibroblasts is truly unexpected. The evidence for transformation is plentiful and conclusive. Cells transformed by overexpression of either the a, b, c, h, or i subunit show increased rates of proliferation and clonogenicity, anchorage-independent growth, loss of contact inhibition, and greater metabolic activity. They also resist entry into apoptosis. In contrast, cells that overexpress any of the other seven eIF3 subunits are not transformed and resemble the parental NIH3T3 cells, except those that overexpress the e and f subunits, which moderately inhibit cell growth and proliferation. It is noteworthy that all of the stable cell lines generated here are isogenic except for the cDNA encoding the eIF3 subunit. The cDNAs are all expressed from the same promoter and are integrated into the same site in the chromosomes. Therefore, the different phenotypes observed are due to the expression of the eIF3 subunit not to effects caused by differences in integration sites.
Of the five transforming subunits, four (a, b, c, and i) are so-called “core” subunits whose homologs are found in the budding yeast, Saccharomyces cerevisiae (8, 20). The genes encoding these subunits are essential in yeast, and together with subunits g and j, are thought to comprise the eIF3 complex capable of functioning in yeast translation (39). In contrast, subunit h has no homolog in S. cerevisiae, although its homolog is found in the eIF3 complex from the fission yeast, Schizosaccharomyces pombe (14). A priori, how overexpression of just any one of the subunits affects the phenotype of the cell is not obvious.

One possible explanation for how overexpression of a single eIF3 subunit causes malignant transformation is that it leads to increased eIF3 activity, which in turn stimulates the translation of mRNAs involved in cell proliferation. The increased eIF3 activity could be due either to higher levels of the eIF3 complex, to altered eIF3 phosphorylation, or to the sequestration of negative regulators of eIF3. Indeed, a 40–50% -fold increase in the level of eIF3a, eIF3b, eIF3f eIF3h, and eIF3j, thought to be representative of the eIF3 complex, is seen in the eIF3a, eIF3b, and eIF3c cell lines, whereas the other cell lines show only a very small increase or no increase at all. However, the translational apparatus in general is not elevated, as the levels of eIF4G, eIF4B, eIF4E, 4E-BP1, eIF2α, and phospho-eIF2α do not change in the transformed cell lines. This suggests that the increase in eIF3 is specific for this translational component. How overexpression of a single eIF3 subunit leads to an increase in the (presumably) whole complex is not apparent.

The three transforming subunits that raise the eIF3 level cannot each be limiting for eIF3 assembly. However, the de novo assembly of eIF3 is almost certainly dynamic, and higher levels of just one of a number of subunits might stimulate the process of assembly, leading to higher levels of the complex. It is noteworthy that the subunits that raise eIF3 levels are the largest subunits, being perhaps the most difficult to assemble into the eIF3 complex. Unfortunately, little is known about the regulation of eIF3 components or their assembly in mammalian cells.

A second way in which eIF3 activity might be stimulated is through phosphorylation. eIF3 is phosphorylated on a number of different subunits, and increased phosphorylation correlates with stimulation of protein synthesis. Overexpression of a subunit might affect certain protein kinases, leading to altered phosphorylation states of eIF3. However, the sites of phosphorylation and the effects of such modifications on eIF3 activity have not yet been elucidated. Additional experiments are required to assess whether or not overexpression of the transforming subunits specifically alters the phosphorylation state and specific activity of eIF3.

A third possible way to affect eIF3 activity is to interfere with a negative regulator of eIF3. The human P56 protein, stimulated by interferon, binds to eIF3e and inhibits initiation (40). One might predict that overexpression of eIF3e could inactivate P56 by forming an eIF3e-P56 complex outside of eIF3, thereby preventing P56 down-regulation of eIF3 activity. Although this hypothetical example provides a mechanism that could apply to the transforming subunits, it does not apply to eIF3e, because eIF3e overexpression leads to inhibition of protein synthesis, not stimulation. Because there are no other known regulators of eIF3 activity comparable to P56, this mechanism remains merely hypothetical.

The one or more mechanisms whereby overexpression of eIF3h and eIF3i stimulates protein synthesis are not obvious. Neither causes an increase in eIF3 level. eIF3h is dispensable in Arabidopsis but has been implicated in the translation of specific mRNAs containing upstream open reading frames, suggesting a role in re-initiation or translational control (19). eIF3i is phosphorylated on a tyrosine residue (41) and is reported to be a substrate of the TGFβ-type 2 receptor (42). A further possibility is that eIF3h and eIF3i (or even the other transforming subunits) affect an aspect of cell metabolism other than protein synthesis. For example, eIF3e is reported to associate with the proteasome in S. pombe, thereby affecting protein degradation (48). Further experimentation is necessary to determine the relevance of these facts and to elucidate how these subunits affect cell proliferation.

Most of the available data leads us to conclude that eIF3 activity is likely enhanced in the transformed cell lines, in three cases because of the increased level of eIF3, and possibly because of changes in phosphorylation status. Unfortunately, a direct measure of eIF3 activity in cells or their lysates is not possible, because suitable methods are not available. Purification of the eIF3 for assays in vitro with other purified initiation components (43) is laborious and actually not very useful; such assays are inefficient and likely would not detect subtle changes in eIF3 specific activity. However, we show that in vivo global protein synthesis is stimulated significantly in the transformed cell lines (30–45%) compared with the control cell line. Furthermore, non-transforming subunits do not affect the translation rate as much (0–20% stimulation), or in the case of the eIF3e and eIF3f cells, actually inhibit protein synthesis. Nevertheless, stimulation of protein synthesis may not be the actual cause of malignancy but rather may be the consequence of the faster growth exhibited by the transformed cells. We therefore measured the translational efficiencies of a number of mRNAs whose products are implicated in cell proliferation, namely cyclin D1, c-Myc, fibroblast growth factor 2, and ornithine decarboxylase. The stimulation of initiation rates, shown by the presence of mRNAs in larger polysomes, is seen with these mRNAs but not with the control glyceraldehyde-3-phosphate dehydrogenase mRNA. We suggest that transforming subunit overexpression leads to the preferential stimulation of so-called “weak” mRNAs whose products promote cell growth, and that these products cause the malignant transformation of NIH3T3 cells. A similar mechanism of action has been proposed for other initiation factors that exhibit transforming activity, e.g. eIF4E, eIF4G, and non-phosphorylatable eIF2α (1, 5).

Failure to down-regulate protein synthesis leads to an overproduction of oncogenic proteins, resulting in malignant transformation (reviewed in Refs. 1 and 5). The model is based on the finding that mRNAs encoding oncogenic proteins such as growth factors and transcription factors are weak competitors with most other mRNAs for the translational apparatus in cells. When global protein synthesis rates are somewhat repressed, the “weak” mRNAs are very inefficiently translated; in contrast,
Oncogenic Role of Five Human eIF3 Subunits

when translation rates are raised, the same class of mRNAs are stimulated much more than the other mRNAs (44). Therefore, growth control is very sensitive to the activity of the translational apparatus and depends on its partial repression to produce low levels of oncogenic proteins. Our results suggest that the rate of protein synthesis is sensitive to the level or activity of eIF3, as is also true for levels of eIF4E, eIF4G, and ternary complexes (5, 45–47). However, it is not known if eIF3 activity is rate-limiting for protein synthesis in non-transformed NIH3T3 cells, and a rigorous demonstration that eIF3 activity is indeed elevated in the transformed cell lines and is directly responsible for the transformation has not yet been achieved. Nevertheless, the results reported here suggest that eIF3 may play an important role in the regulation of initiation rates, a view that is only recently becoming appreciated. The addition of eIF3 to the list of initiation factors that may influence translation rates provides still another potential therapeutic target of intervention in the control of malignant growth.

REFERENCES

1. Rosenwald, I. B. (2004) Oncogene 23, 3230–3247
2. De Benedetti, A., and Rhoads, R. E. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 8212–8216
3. Lazaris-Karatzas, A., Montine, K. S., and Sonenberg, N. (1990) Nature 345, 544–547
4. Avdulov, S., Li, S., Michalek, V., Burrichter, D., Peterson, M., Perlman, D. M., Manivel, J. C., Sonenberg, N., Yee, D., Bitterman, P. B., and Polonovska, V. A. (2004) Cancer Cell 535–563
5. Mamanie, Y., Petroulakis, E., Rong, L., Yoshida, K., Ler, L. W., and Sonenberg, N. (2004) Oncogene 23, 3172–3179
6. Ruggiero, D., Montanaro, L., Ma, L., Xu, W., Londei, P., Cordon-Cardo, C., and Pandolfi, P. P. (2004) Nat. Med. 10, 484–486
7. Wendel, H. G., De Stanchina, E., Fridman, J. S., Malina, A., Ray, S., and Derynck, R. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 1343–1351
8. Hershey, J. W. B., and Merrick, W. C. (2000) in Gene Expression (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., eds) pp. 33–88, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
9. Koromilas, A. E., Roy, S., Barber, G. N., Katze, M. G., and Sonenberg, N. (1992) Science 257, 1685–1689
10. Donze, O., Jagus, R., Koromilas, A. E., Hershey, J. W., and Sonenberg, N. (1995) EMBO J. 14, 3828–3834
11. Raught, B., Gingras, A. C., James, A., Medina, D., Sonenberg, N., and Rosen, J. M. (1996) Cancer Res. 56, 4382–4386
12. Morris-Desbois, C., Retey, S., Ferro, M., Garin, J., and Jalnin, P. (2001) J. Biol. Chem. 276, 45988–45995
13. Mayeur, G. L., Fraser, C. S., Peiretti, F., Block, K. L., and Hershey, J. W. (2003) Eur. J. Biochem. 270, 4133–4139
14. Zhou, C., Arslan, F., Wee, S., Krishnan, S., Ivanov, A. R., Oliva, A., Leithwood, J., and Wol, D. A. (2005) BMC Cell. Biol. 3, 14
15. Unbehaun, A., Borukhov, S. I., Hellen, C. U., and Pestova, T. V. (2004) Genes Dev. 18, 3078–3093
16. Siritidechadilok, B., Fraser, C. S., Hall, R. J., Doudna, J. A., and Nogales, E. (2005) Science 310, 1513–1515
17. Dong, Z., and Zhang, J. T. (2003) Mol. Biol. Cell 14, 3942–3951
18. Dong, Z., Liu, L. H., Han, B., Pincheira, R., and Zhang, J. T. (2004) Oncogene 23, 3790–3801
19. Kim, T. H., Kim, B. H., Yalahom, A., Chamovitz, D. A., and von Arnim, A. G. (2004) Plant Cell 16, 3341–3356
20. Phan, L., Zhang, X., Asano, K., Anderson, J., Vornlocher, H. P., Greenberg, J. R., Qin, J., and Hinnebusch, A. G. (1998) Mol. Cell. Biol. 18, 4935–4946
21. Pincheira, R., Chen, Q., and Zhang, J. T. (2001) Br. J. Cancer 84, 1520–1527
22. Bachmann, F., Banziger, R., and Burger, M. M. (1997) Cancer Res. 57, 988–994
23. Delas, A., Torhorst, J., Bachmann, F., Banziger, R., Schultheiss, E., and Burger, M. M. (1998) Cancer 83, 1376–1383
24. Chen, G., and Burger, M. M. (1999) Int. J. Cancer 84, 95–100
25. Lin, L., Holbro, T., Alonso, G., Gerosa, D., and Burger, M. M. (2001) J. Cell. Biochem. 80, 483–490
26. Rothe, M., Ko, Y., Albers, P., and Wernert, N. (2000) Am. J. Pathol. 157, 1597–1604
27. Joseph, P., Lei, Y. X., Whong, W. Z., and Ong, T. M. (2002) Cancer Res. 62, 703–707
28. Joseph, P., Lei, Y. X., and Ong, T. M. (2004) Mol. Cell Biochem. 255, 93–101
29. Nupponen, N. N., Porkka, K., Kakkola, L., Tanner, M., Persson, K., Borg, A., Isola, J., and Visakorpi, T. (1999) Am. J. Pathol. 154, 1777–1783
30. Nupponen, N. N., Isola, J., and Visakorpi, T. (2000) Genes Chromosomes Cancer 28, 203–210
31. Saramaki, O., Willi, N., Brinkmann, A. O., Gallahan, D., and Callahan, R. (1997) Oncogenes 6, 155–158
32. Marchetti, A., Buttiatta, F., Pellegrini, S., Bertacca, G., and Callahan, R. (2001) Int. J. Oncol. 18, 175–179
33. Buttiatta, F., Martella, C., Barassi, F., Felici, L., Agliano, S., and Rosini, S., D’Antuono, T., Chella, A., Mucilli, F., Sacco, R., Mezzetti, A., Cuccurullo, F., Brinkmann, A. O., and Visakorpi, T. (2005) Clin. Cancer Res. 11, 3198–3204
34. Fraser, C. S., Lee, J. Y., Mayeur, G. L., Bushell, M., Doudna, J. A., and Hershey, J. W. (2004) J. Biol. Chem. 279, 8946–8956
35. Brown-Luedi, M. L., Meyer, L. J., Milburn, S. C., Yau, P. M., Corbett, S., and Hershey, J. W. (1982) Biochemistry 21, 4202–4206
36. Nielsen, K. H., Valkas, L., Sykes, C., Ivotovsky, I., and Hinnebusch, A. G. (2006) Mol. Cell. Biol. 26, 2984–2998
37. Hui, D. J., Bhasker, C. R., Merrick, W. C., and Sen, G. C. (2003) J. Biol. Chem. 278, 39477–39482
38. Rush, J., Moritz, A., Lee, K. A., Guo, A., Goss, V. L., Spek, E. J., Zhang, H., Zha, X. M., Polakiewicz, R. D., and Comb, M. J. (2005) Nat. Biotechnol. 23, 94–101
39. Choy, L., and Derynck, R. (1998) J. Biol. Chem. 273, 31455–31462
40. Benne, R., and Hershey, J. W. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 3305–3309
41. Chang, H., Langer, P. J., and Lodish, H. F. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 3206–3210
42. De Benedetti, A., and Graff, J. R. (2004) Oncogene 23, 3189–3199
43. Gingras, A. C., Raught, B., and Sonenberg, N. (1999) Annu. Rev. Biochem. 68, 913–963
44. Zimmer, S. G., DeBenedetti, A., and Graff, J. R. (2000) Anticancer Res. 20, 1343–1351
45. Yen, H.-C. S., Gordon, C., and Chang, E. C. (2003) Cell 112, 207–217

5800 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 282 • NUMBER 8 • FEBRUARY 23, 2007