Translational Regulation of Ribonucleotide Reductase by Eukaryotic Initiation Factor 4E Links Protein Synthesis to the Control of DNA Replication*

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Ribonucleotide reductase synthesizes dNDPs, a specific and limiting step in DNA synthesis, and can participate in neoplastic transformation when overexpressed. The small subunit (ribonucleotide reductase 2 (RNR2)) was cloned as a major product in a subtraction library from eukaryotic initiation factor 4E (eIF4E)-transformed cells (Chinese hamster ovary-4E (CHO-4E)). CHO-4E cells have 20–40-fold elevated RNR2 protein, reflecting an increased distribution of RNR2 mRNA to the heavy polysomes. CHO-4E cells display an altered cell cycle with shortened S phase, similar to cells selected for RNR2 overexpression with hydroxyurea. The function of ribonucleotide reductase as a checkpoint component of S progression was studied in yeast in which elevated eIF4E rescued S-arrested rnr2–68° cells, by increasing recruitment of its mRNA to polysomes. Crosses between rnr2–68° and mutant eIF4E (cdc33–1 rnr2–68°) engendered conditional synthetic lethality, with extreme sensitivity to hydroxyurea and the microtubule depolymerizing agent, benomyl. The double mutant (cdc33–1 rnr2–68°) also identified a unique terminal phenotype, arrested with small bud and a randomly distributed single nucleus, which is distinct from those of both parental single mutants. This phenotype defines eIF4E and RNR2 as determinants in an important cell cycle control transition in yeast. Overexpression of eIF4E causes deregulated cell growth and malignant transformation of rodent and human cells (9–11). Hence, the mechanism of transformation by eIF4E presents an important and challenging biological phenomenon. Furthermore, eIF4E is elevated in common human neoplasms, such as carcinomas of the breast and the head and neck (12, 13). Hence the translation of certain proto-oncogene or growth factor transcripts, which normally are translationally repressed, becomes preferentially increased by an excess eIF4E. Accordingly, a few of these have been identified, e.g. c-Myc and ornithine decarboxylase (ODC) (reviewed in Refs. 14 and 15). However, a systematic identification of the transcripts of which the translation is increased by the excess eIF4E would represent a major step forward in understanding the role of dysregulation of protein synthesis in cancer etiology. We have addressed this task with the construction of a subtraction cDNA library from the heavy polysomes of CHO cells overexpressing eIF4E (CHO-4E). From an initial screening, we identified an abundantly represented product corresponding to the small subunit of ribonucleotide reductase (RNR2).

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1 The abbreviations used are: eIF4E, eukaryotic initiation factor 4E; RNR, ribonucleotide reductase; CHO, Chinese hamster ovary; FGF, fibroblast growth factor; ODC, ornithine decarboxylase; kb, kilobase(s); HU, hydroxyurea; YPAd, yeast extract-peptone-dextrose; YPGal, yeast extract-peptone-galactose; UTR, untranslated region.
restrictive temperature with large budded cells (19, 21). Agents that inhibit dNTPs synthesis, such as methotrexate, are used to inhibit replication of cancer cells; conversely, elevated RNR may hasten the cell division. Accordingly, RNR2 was recently recognized as a powerful transforming agent in cooperation with a variety of oncogenes in mammalian cell lines (22, 23). RNR2 was also identified as an overexpressed product in a panel of breast carcinomas (24).

We propose that increased expression of RNR2 in cells with elevated eIF4E represents an important avenue by which eIF4E can induce malignant transformation. Moreover, the evidence herein for translational regulation of RNR2 constitutes perhaps the most direct link between a central player in protein synthesis (eIF4E) and a key enzyme in DNA synthesis.

EXPERIMENTAL PROCEDURES

Cell Lines and Tissue Culture—Chinese hamster ovary cells stably transfected with BK or BK-4E episcopal vectors were cultured as described previously (10).

DNA Synthesis and Cell Cycle Synchronization—For synchronization experiments, CHO-BK and CHO-4E cells were arrested in G0/G1 or in early G2, with some modifications of the published procedure (25). In brief, aliquots of 106 cells grown on 60-mm plates were arrested at G0/S with 5 μg/ml aphidicolin (Sigma) for 18 h, released from the block with three washes of fresh medium, and allowed to grow for 12 h. Finally, the cultures were serum-starved for 12 h. This treatment resulted in greater than 80% of the cells arrested in G0/S, or early G1 phase of the cell cycle. DNA synthesis was measured by incorporation of [6-3H]thymidine (15 Ci/mmol; NEN Life Science Products) added directly to the cultures 1 h before harvest, washed, and dried, and resuspended in 200 μl of DEPC-H2O. For Northern blots, the RNA was denatured with glyoxal/MeSO, separated on a 1.3% agarose gel and transferred to Immobilon-N membrane (Millipore Co.). After hybridization, the blots were exposed to autoradiography film for 1–4 days.

RNR2 transcripts were also identified in extracts of CHO-BK and CHO-4E cells by Northern analysis (Fig. 4). An RNR2 probe was obtained by PCR using oligonucleotide primers (26), RNR2 (5′GCGAATTCATCATGGGCAATAAGGAATAATGCGG3′ and 5′TCGACTTTTGGGXXGXXGXXG3′) and RNR1 (5′GCGAATTCATCATTGGGCTACAGGACTGTTCAAA3′ and 5′TCGACTTTTGGGXXGXXGGXXG3′) and Klenow polymerase was used for second-strand synthesis, and the same set of primers was then cycled in cycles of polymerase chain reaction to slightly amplify the cDNA. The polymerase chain reaction profile was 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 5 min. After 30 cycles there was no significant increase in PCR fragment size.

Cloning of the Subtraction cDNA Library—The P100 fraction was depleted of hydroxyurea or benomyl were made by adding the indicated amount of hydroxyurea or benomyl to YPAD or YPGal agar after autoclaving. Yeast transformation was carried out essentially as described (29) with 10% formaldehyde for 3 min. The membrane was prehybridized with 5× SDS, 0.5 M NaHPO4 (pH 7.4), 10 mM EDTA at 45 °C for 3 h and hybridized overnight at the same temperature. The RNA was then washed, ethanol- washed, hybridized overnight at 45 °C in the presence of RNA from the S100 (fewer than 5 ribosomes), the supernatant was mixed with an equal volume of 3% CETAB (cetyl-trimethylammonium bromide, Sigma). RNA was precipitated by centrifugation at 30,000 × g. The next steps were similar for extraction of RNA from S100 and P100: the precipitates were redissolved in 200 μl of 5 M guanidine-HCl and 0.5 M NaHPO4/0.5 M NaCl/0.1% Triton X-100/0.5% sodium deoxycholate. The RNA was electrophoretically separated on a 1.3% agarose gel composed with 0.5% formaldehyde and stained with ethidium bromide. RNA bands were detected by UV fluorescence and quantitated by densitometry of the gel. The next steps were similar for extraction of RNA from S100 and P100: the precipitates were redissolved in 200 μl of 5 M guanidine-HCl and 0.5 M NaHPO4/0.5 M NaCl/0.1% Triton X-100/0.5% sodium deoxycholate. The RNA was electrophoretically separated on a 1.3% agarose gel composed with 0.5% formaldehyde and stained with ethidium bromide. RNA bands were detected by UV fluorescence and quantitated by densitometry of the gel.

Fluorescence Microscopy and Fluorescence-activated Cell Sorting Analysis—Exponentially growing cultures in YPAD liquid medium at 22 °C was shifted to 30 °C. After the cells arrested, cellular DNA was visualized by staining with 4′,6-diamidino-2-phenylindole as described (30). Cells were prepared for fluorescence-assisted cell sorting by staining with propidium iodide as described (31).

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RESULTS

Excess eIF4E Elicits a Selective Redistribution of mRNA Classes in the Heavy Polysomal Fractions—We previously reported that overexpression of eIF4E in CHO (or continuous rat embryo fibroblasts) cells resulted in a transformed phenotype, which included loss of contact inhibition, shortening of generation time, and growth in soft agar (11). The level of eIF4E was increased 7-fold (uninduced), and 20-fold following induction of the promoter with tetrachlorodibenzodioxin, compared with control CHO-BK cells. Consistent with this elevation in eIF4E, the overall rate of protein synthesis was increased by 30 and 50% in CHO-BK versus CHO-4E cells (Fig. 1A). This result is consistent with the model of competition for mRNA recruitment to the ribosomes mentioned above and also forms the theoretical basis for generation of an enriched cDNA library.

Briefly, poly(A)-containing RNA from the heavy polysomes of CHO-4E cells provided the source for synthesis of the first-strand of cDNA; after hybridization with an excess of polysomal mRNA from control cells (CHO-BK), common transcripts were physically removed (see under “Experimental Procedures”). To simplify the process, instead of collecting multiple gradient fractions, we extended the centrifugation time from 2 (Fig. 1A) to 4 h to sediment particles corresponding to ≥6 ribosomes. The ribosome pellet, termed P100, was used to generate the cDNA library. The rest of the gradient, containing particles with a sedimentation coefficient of five or fewer ribosomes, was termed S100 and was used as a control in subsequent experiments. An example of this fractionation is shown in Fig. 1B, where the distribution of FGF-2 mRNA and ODC, another eIF4E-dependent mRNA (33), in the P100 and S100 fractions of CHO-BK versus CHO-4E is displayed. The total amount of FGF-2 or ODC mRNA was the same in CHO-BK and CHO-4E cells, when S100 and P100 fractions are integrated. However, there is a reciprocal redistribution of FGF-2 (and ODC) mRNAs in the S100 and P100 of these cells (Fig. 1B). For comparison, the Northern blot was probed for cyclin-D1 mRNA, which was similar in CHO-BK and CHO-4E (Fig. 1B, right panel), indicating equivalent expression and polysomal distribution.

**RNR2 Is a Major Protein Identified in a Subtraction Library from the Heavy Polysomes of CHO-4E**—The cDNA library described above was initially tested for enrichment by microarray display and quantitative polymerase chain reaction, using primers for typical strong mRNAs (e.g., glyceraldehyde-3-phosphate dehydrogenase) versus weak mRNAs (e.g., FGF-2 and vascular endothelial growth factor) previously known to be preferentially recruited by the excess eIF4E (26, 34). Following these preliminary tests (data not shown), we analyzed the cDNA inserts by restriction digestions and partial sequencing. Among the first identified cDNA inserts, there were several independent clones corresponding to the RNR2 mRNA of golden hamster (Mus auratus; 97% homology). To confirm that RNR2 is a translationally regulated product in cells transformed with eIF4E, we probed the Northern blot in Fig. 1B with a partially sequenced clone, corresponding to nucleotides 2212–3064 of RNR2 of M. auratus. The Northern blot revealed a transcript of the expected size (~3 kb) (35), and it demonstrated a reciprocal redistribution of the RNR2 mRNA in the P100 and S100 fractions (Fig. 2A). There was a 6-fold increase in RNR2 mRNA in the P100 of CHO-4E compared with CHO-BK, as measured by densitometric analysis and by reprobing the slot-blot in Fig. 1A (data not shown). The total amount of RNR2 mRNA was similar in the two cell lines (Fig. 2A, right panel). This result proves that RNR2 mRNA is engaged for translation initiation far more efficiently in CHO-4E cells and that it may fall into the category of translationally repressed transcripts.
increase in RNR2 (data not shown). It seems plausible that the shorter S phase, completed in 14 h. These cells have a 5-fold reduction in generation time of CHO-4E cells. Similarly, cells selected for elevated expression of RNR2 by progressive selection in hydroxyurea (CHO-HU in Fig. 3) also displayed a shorter S phase, completed in 14 h. These cells have a 5-fold increase in RNR2 (data not shown). It seems plausible that the excess RNR2, the limiting component of RNR in mammals, could speed up the replication period by making a larger pool of dNTPs available to the DNA replication machinery. However, these data cannot exclude the possibility of activation of an additional pathway that may facilitate the DNA replication process.

CHO-4E Cells Are Naturally Resistant to Hydroxyurea—HU specifically inactivates RNR by scavenging the tyrosyl radical at the catalytic site of RNR2. Overexpression of RNR2, but not of RNR1, was shown to increase cell resistance to HU (16, 35). We thus tested whether CHO-4E cells were more tolerant to growth inhibition by HU. The cells were grown in 24-well dishes (in duplicates) and treated for 8 h with increasing concentrations of HU. The cells were then incubated for 17 h in presence of 10 μCi of [3H]thymidine to measure the inhibition of DNA synthesis by HU (see under “Experimental Procedures”). The calculated LD50 for CHO-BK was near 0.1 mM, whereas it was 1 mM for CHO-4E (data not shown). This confirmed that CHO-4E cells are more resistant to HU, consistent with their elevated expression of RNR2.

Overexpression of eIF4E Restores Growth to a rnr2–68 Yeast Strain—The alteration in cell cycle caused by eIF4E prompted us to determine whether elevated eIF4E generally increases RNR expression in other systems, with corresponding effects on the cell cycle. Thus, we took advantage of a S. cerevisiae strain, rnr2–68, harboring a ts mutation in RNR2. At the restrictive temperature (34 °C) this strain stops growing and arrests in S phase with abnormally large buds. At the permissive temperature (23 °C), the cells are viable with the majority traversing the S phase slowly. This strain was transformed with a vector expressing hemagglutinin-tagged eIF4E (CD33) under the control of a GAL1 promoter (YCplF15-CDC33), or with a control vector lacking CDC33. Functional activity of this hemagglutinin-tagged eIF4E was confirmed in vivo by its ability to complement cdc33–1 ts mutant at 37 °C (data not shown). Trp+ YCplF15-CDC33 transformants were streaked onto synthetic medium containing 2% galactose, to induce the expression of eIF4E. As shown in Fig. 4A, overexpression of eIF4E restored growth to the rnr2–68 strain at nonpermissive temperature. By contrast, rnr2–68 containing YCplF15 (control vector) was unable to grow at 34 °C. This suppression of heat sensitivity of rnr2–68 may be due to either an increase in the expression of rnr2–68 allele due to excess eIF4E or activation of an alternate pathway that allows cells to bypass the mutation.

At permissive temperature, the doubling time of the mutant, rnr2–68, is 8 h. The doubling time was reduced by approximately 50% when overexpression of eIF4E was induced with galactose. Thus, rnr2–68 grows better even at permissive temperature when eIF4E is overexpressed.

The Double Mutant, cdc33–1 rnr2–68, Shows Conditional Synthetic Lethality—To further elucidate the relation between eIF4E and RNR2 expression, we constructed a cdc33–1 rnr2–68 double mutant by crossing the single mutants (see under “Experimental Procedures”). After tetrad dissection, all double mutants obtained from tetratype and nonparental di- type gave rise to very small colonies compared with the single mutants (Fig. 4B). At permissive temperature (20 °C), double mutant cells showed a very slow growth phenotype with extreme sensitivity to HU (unable to grow on plates containing 1 mM of HU) and to the microtubule depolymerizing drug benomyl (unable to grow on plates containing more than 5 μg/ml). The wild-type parental strain (CRY1) is resistant up to a concentration of 200 mM HU and 15 μg/ml benomyl. The single mutants, cdc33–1 and rnr2–68, can grow up to 34 °C; the double mutant showed conditional synthetic lethality at 28 °C in rich medium (Fig. 4C). Fig. 4D shows that the double mutant
by modulating the expression of RNR2 as one of its downstream effectors. However, it is likely that some other cell components, in addition to RNR, are also affected by the reduction of functional eIF4E. In the double mutant, the smaller bud size and random distribution of the undivided nucleus in either mother or daughter cells are indicative of this (Fig. 4D).

In contrast, the typical S phase-arrested rnr2-68 shows abnormally large-budded cells with nuclei being either in the mother cell or near the bud neck, but never in the daughter cell.

eIF4E Regulates the Expression of RNR2 at the Translational Level in Yeast—The evidence provided above suggested that the expression of RNR2 is regulated by eIF4E in yeast as well. In order to see whether this control by eIF4E is at the transcriptional and/or translational level, we tested the in vivo effects of overexpression of eIF4E on the message level of RNR2 and on the recruitment of RNR2 mRNA onto the polysomes. The polysomal fractions were obtained from an exponentially growing culture of a wild-type strain containing either control vector or the eIF4E overexpressing vector (YCpIF15-CDC33), grown in minimal medium without Trp containing 2% raffinose + 2% galactose. The Northern blot revealed a redistribution of the RNR2 mRNA in the P100 and S100 fractions in the CDC33-overexpressing strain compared with the control. Fig. 5A shows that there is a 6-fold increase in RNR2 mRNA in the P100 fraction of eIF4E-overexpressing strain compared with that of CRY1-control, as measured by densitometric analysis of this and another slot-blot (data not shown). However, the total amount of RNR2 mRNA did not change in the two strains (data not shown). This indicates that there is an increase in the translation initiation rate of RNR2 in eIF4E-overexpressing cells.

We also examined the effects of depletion of functional eIF4E (CDC33) on the expression of RNR2. The congenic cdc33-1 ts strain that arrests in G1 at 34 °C or above (8) was tested in a temperature shift experiment. When an exponential culture of this strain grown in YPAD was shifted from 23° to 37 °C, RNR2 protein was drastically reduced within 1–2 h of incubation, whereas the level of α-tubulin was unaffected (Fig. 5B). [35S]Methionine incorporation showed that the shift to nonpermissive temperature did not result in gross changes in the total protein synthesis pattern during the first hour (data not shown). This indicates again that the loss of eIF4E function results in discreet changes in protein synthesis (like RNR2) rather than global, at least initially. There were also no observable changes in the level of RNR2 or RNR1 mRNAs (Fig. 5B, bottom panel), suggesting that the specific decrease in the synthesis of RNR2 protein upon reduction of eIF4E activity occurs at a posttranscriptional level.
solved with the finding that the S phase transit period was shortened (by 3–4 h) in CHO-4E, demonstrating that these cells can complete DNA replication faster than normal. At this point, we do not know precisely how an increase in RNR is translated into a corresponding increase in the rate of DNA replication in CHO-4E. However, cells selected for elevated expression of RNR2 by progressive selection in hydroxyurea (CHO-HU) also showed a shorter S phase. Because synthesis of deoxynucleotides is rate-limiting for DNA synthesis in most organisms, an excess of RNR2 may allow for a faster replication of the genome, especially considering that replication in eukaryotes proceeds simultaneously from multiple origins.

Translational regulation of RNR2 is well known for clam and sea urchin eggs, in which fertilization triggers its synthesis as one of the most abundant products of maternal mRNAs (36). Translational regulation of RNR has not been reported in other systems, except for the finding of an instability determinant in the 3'-UTR of RNR2 mRNA (44). However, this is the first report of a specific translational enhancement by eIF4E, classifying RNR2 as a weak mRNA and suggesting modulation by its 5'-UTR. The function of eIF4E is to nucleate the assembly of preinitiation complexes at the mRNA 5'-UTR, although the 3'-UTR can also participate in this process (45). Our data indicate that translation of RNR2 is specifically facilitated by excess eIF4E. This was demonstrated by an analysis of polysomal profile of RNR2 mRNA in cells overexpressing eIF4E. Interestingly, suc22”, the RNR2 homolog of S. pombe, is normally expressed as a 1.5-kb transcript, but upon treatment with hydroxyurea a 1.9-kb transcript with an extended 5'-UTR is induced (46), suggesting that it may be translationally regulated as well. The same phenomenon was observed for the RNR4 gene, a RNR2 homolog of S. cerevisiae (47, 48).

We also showed that inducible overexpression of eIF4E rescues viability in a yeast strain harboring a ts mutation in the small subunit of RNR (rnr2–68) at the restrictive temperature. Overexpression of eIF4E also compensated growth defects of the rnr2–68 mutant as evidenced by a 50% reduction in generation time. Our experiments do not rule out the possibility that excess eIF4E may also result in an increase in RNR1 in this strain. However, note that overexpression of RNR1 in the rnr2–68 strain could not restore growth at 34 °C,2 demonstrating that the intracellular RNR1 level is not limiting in this strain (see also in Ref. 19). Previously, RNR1 was reported to be cell cycle-regulated at the transcriptional level, fluctuating 15–30-fold (18), whereas the RNR2 transcript showed no more than a 2-fold change during the cell cycle. It would then follow that RNR2 must be translationally regulated during the cell cycle, in order to be stoichiometric with RNR1. In this context, we should recall that eIF4E activity fluctuates during the cell cycle (25), and conversely, eIF4E was originally cloned as a ts mutant controlling cell cycle progression in yeast (CDC33, Ref. 8). Taken together with the results described here, it is likely that transcriptional and translational regulation of the RNR genes is an ancient, evolutionarily conserved mechanism. This is further supported by the fact that the double mutant of cdc33–1 and rnr2–68 could not grow at temperatures above 28 °C, suggesting conditional synthetic lethality. We also reported that the double mutant presents with a cell cycle arrest that is different from either of the single mutants. The fact that cdc33–1 rnr2–68 double mutant did not arrest in early G1 obviates the possibility that the phenotype seen in the double mutant was solely due to a direct effect of loss of functional

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2 M. R. Abid, Y. Li, C. Anthony, and A. De Benedetti, unpublished observation.
In contrast, the single mutant rnr2–68 strain arrests in the middle of S phase with large buds at restrictive temperature, reflecting the fact that RNR first appears in late G1 and reaches its peak in S phase (18, 19). The S phase arrest in the double mutant has a different phenotype from that of rnr2–68, as evidenced by their differences in cell morphology (small buds), distribution pattern of nucleus, and sensitivities to HU and benomyl. These argue in favor of the hypothesis that the double mutant displays a phenotype that depends on both cdc33–1 and rnr2–68 functions. The S phase arrest in the double mutant can then occur by the following models. cdc33–1 cells grow slowly and traverse G1 much slower than wild-type cells even at permissive temperature (8). The decreased translation of the rnr2–68 mRNA (and presumably some other G1 or G2 specific mRNAs) caused by a defective cdc33–1 in the double mutant may further slow down the cells in late G1. This may result in an increase in the cell size that allows the cells to bypass the size checkpoint in G1 and to proceed into S with a very low level of active RNR. Plausibly, this results in the early S phase arrest seen in this strain with small budded cells.

In retrospect, it is not surprising that synthesis of RNR, a key enzyme in DNA replication, should be placed under translational control. In a sense, the step at which synthesis of dNTPs commences is the most direct indication that the cell is metabolically prepared to undertake the expenses of replication. Such an endeavor cannot be limited to the presence of favorable mitogenic signals from the environment, but it also necessarily requires assessing of a replication-competent enzymatic milieu, of which fully active protein synthesis is perhaps the best indicator (49). Similarly, translational control of RNR2 upon DNA damage would be very advantageous because of the rapidity of such a response, which does not require de novo gene expression. Very recently, it has been shown that in addition to transcriptional control of RNR1, a posttranslational component plays an important role in the regulation of RNR activity. Sm1p inhibits dNTP synthesis by binding directly to RNR1 subunit in yeast (50).

Exogenous expression of RNR2 has been recently recognized as a powerful enhancer of transformation in cooperation with several oncogenes (22, 23). Interestingly, many large DNA tumor viruses (herpesviridae and Epstein-Barr virus) encode their own genomic complement of RNR2 (and RNR1 in some cases) rather than relying on the limiting cellular enzyme (51). The RNR2 gene is also frequently amplified in drug-resistant cancers, particularly following treatment with hydroxyurea or MDL101731, another specific inhibitor of RNR2 (52, 53). Furthermore, increased expression of RNR2 is recognized as an early change in ductal carcinomas in situ (24) and in atypical hyperplastic oral lesions (54). Coincidentally, these are the same type of lesions that we have been studying as the earliest pathological abnormalities in which overexpression of eIF4E becomes apparent (38, 55). Thus, we propose that, as the level of eIF4E increases during malignant progression, there is a corresponding increase in RNR2 translation and RNR assembly, leading to accelerated cell replication and increased tolerance to DNA damaging agents. As such, the identification of RNR2 as a major protein increased by excess eIF4E adds a new important player to the limited spectrum of translationally enhanced mRNAs that clearly play a key role in eIF4E-induced cell transformation. The fact that elevated eIF4E, with its corresponding effect on RNR2 expression, may naturally protect cells from common chemotherapeutic drugs adds another reason to design strategies to target eIF4E in cancer therapy.