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Increased expression of eukaryotic translation initiation factors eIF-4E and eIF-2α in response to growth induction by c-*myc*

(delayed early genes/gene expression/protein synthesis)

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ABSTRACT **Although activation of c-*myc* is a critical step in the development of lymphomas and other tumors, its normal function(s) in cell growth remain obscure because few *myc*-regulated genes are known. *myc* expression normally increases in response to mitogens and peaks in G_1 when additional protein synthesis is required for cell-cycle progression. Protein synthesis is controlled by the availability of translation initiation factors, including the mRNA cap binding protein (eIF-4E) and the α subunit of the eIF-2 complex that binds the initiator Met-tRNA. Consequently we examined eIF-4E and eIF-2α for evidence of regulation by c-*myc*. Expression of eIF-4E and eIF-2α correlated with c-*myc* expression in fibroblasts after growth stimulation. In addition, expression of eIF-4E and eIF-2α was increased in *myc*-transformed rat embryo fibroblasts but was not increased in *ras*-transformed cells. Transcription rates of eIF-4E and eIF-2α mRNAs were regulated by c-*myc* in cells expressing an estrogen receptor–Myc fusion protein. Finally, electrophoretic mobility-shift assays identified a sequence element in the eIF-2α promoter, TCCGCATGCGCG, which was specifically retarded by extracts of *myc*-expressing cells. c-*myc* is thought to deregulate the growth of cancer cells by activating transcription, suggesting that specific genes regulated by c-*myc* should also function as oncogenes. In previous studies these translation initiation factors could induce neoplastic growth because overexpression of eIF-4E-transformed cells and inhibition of a suppressor of eIF-2α (eIF-2α kinase) also caused malignant transformation. Our studies suggest that one important biological function of c-*myc* may be to increase cell growth by increasing expression of eIF-4E and eIF-2α.**

Transcriptional activation of c-*myc* by translocation or amplification is a critical step in the development of lymphomas and other tumors. Although c-*myc* is thought to function as a transcriptional activator, its functions in the regulation of cell growth and metabolism are poorly understood because few *myc*-regulated genes have been identified (1–6). After mitogenic stimulation, c-*myc* is maximally expressed during the G_1 phase of the cell cycle (7). During the transition from G_0 to G_1, protein synthesis increases in response to mitogens, and additional protein synthesis is required for cell-cycle progression (8–10). Although numerous studies have demonstrated that growth stimulation increases protein synthesis, little evidence has appeared to link specific transforming genes to an increase in translation.

Two translation initiation factors (eIF-4E and eIF-2α) play important roles in regulating protein synthesis (11, 12). Importantly, quantitative changes in eIF-4E (the mRNA cap binding protein) are known to regulate cell growth because eIF-4E overexpression itself transforms cells to a malignant phenotype (13). Moreover, mutations in the *CDC33* gene (encoding eIF-4E) arrest budding yeast in G_1 (14). Similarly, loss of function of an inhibitor of eIF-2α (eIF-2α kinase) deregulates cell growth (15). The importance of these translation initiation factors in regulating cell growth and the coincidence in timing between increased protein synthesis and increased c-*myc* expression led us to examine the involvement of c-*myc* in regulating expression of translation initiation factors eIF-4E and eIF-2α.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. BALB/c 3T3 cells were provided by C. Stiles, Dana–Farber Cancer Institute, Boston. Rat embryo fibroblasts (REFs) transfected with a plasmid encoding a G418-resistance marker alone (REF-neo) or co-transfected with an expression plasmid for c-*myc* (REF-myc) or *ras* (REF-ras) and the separate neomycin resistance-expressing plasmid were provided by R. Weinberg, Whitehead Institute, Cambridge, MA. Cells expressing a genetic construct encoding a protein that contains the estrogen-binding domain of the estrogen receptor (er) fused to c-*myc* were provided by M. Bishop, University of California Medical School, San Francisco, CA (6, 16). The Myc–er fusion protein has low c-*myc* activity in the absence of estradiol, but c-*myc* function can be induced by adding estradiol to BALB/c 3T3 cells expressing this construct (myc–er cells). Estrogen-stimulation experiments were done as described (6, 16).

RNA Blot Analysis and Run-off Assays. Total cellular RNA (17) was size-fractionated on formaldehyde-agarose gels (10 μg per lane), transferred to Hybond-N nylon matrices, and crosslinked by UV light. Filters were hybridized overnight at 42°C with eIF-4E (13), eIF-2α (11), c-Myc, tubulin, and human EF-1α (18) cDNA fragments ^{32}P-labeled by the Klenow reaction using random priming.

To assess stability of eIF-4E and eIF-2α mRNAs, actinomycin D (5 μg/ml) was added to control or estradiol-stimulated myc–er cells. mRNA was harvested and analyzed at the indicated times. For run-on experiments, nuclei from resting myc–er cells or myc–er cells stimulated by estradiol (0.5 μM) for 4 hr were harvested, and nuclear run-on assays were done as described (19, 20).

Electrophoretic Mobility-Shift Assays (EMSAs). Nuclear extracts were prepared from 10^8 HeLa, REF-Neo, REF-myc, and HL-60 cells during logarithmic growth for EMSAs (21). Gel-shift oligonucleotides included the following: E, a trimer of the E box motif (TGTCACTTGAC) identified between −561 and −550 in the eIF-2α promoter; PAL oligonucleotide, a probe containing the directly repeated palindromic se-

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Abbreviations: NRF, nuclear respiratory factor; EMSA, electrophoretic mobility-shift assay; er, estrogen receptor; PDGF, platelet-derived growth factor; REF, rat embryo fibroblast.

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quences from −58 to −24 (GCTTGCGCATGCGAGGAG-GTTCCGCATGCGCGGTG) of the eIF-2α promoter, and μ, a mutant PAL oligonucleotide (GCTTGCTCATCCGAG-GAGGTTCCTCATCCGCGGTG). Binding reactions included 5 μg of the indicated extracts (neo, REF-neo; myc, REF-myc) or the indicated extracts plus a 100-fold excess of nonradioactive probe. We further compared binding in the presence of herring sperm DNA and poly(dI·dC) as nonspecific competitors.

RESULTS

We first examined the expression of c-Myc, eIF-4E, and eIF-2α in serum-stimulated fibroblasts (Fig. 1). After growth stimulation by either serum or platelet-derived growth factor (PDGF), an early rise in c-Myc mRNA began at 0.5 hr, reached a maximum at 2 hr, and continued until 18 hr. Increases in c-Myc were closely followed by increases in eIF-4E expression (Fig. 1, 2–18 hr). eIF-2α mRNA also increased in response to serum stimulation and to PDGF, beginning at 2 hr and continuing until 18 hr. In contrast, expression of mRNAs for elongation factor EF-1α and tubulin was constant. The biphasic increase in c-Myc seen in this experiment has been reported (22).

We then examined eIF-4E and eIF-2α mRNA levels in *myc*-transfected REF cells (REF-myc) (Fig. 2). Elevated eIF-4E and eIF-2α mRNA levels were seen in REF-myc cells cultured in either high or low serum. In contrast, eIF-4E and eIF-2α expression was significantly increased in *ras*-transfected REF cells (REF–ras) only when growing in 10% serum, a condition in which c-*myc* expression was strongly increased. Expression of elongation factor EF-1α did not vary significantly under these conditions.

To correlate eIF-4E and eIF-2α expression with *myc* function, we used BALB/c 3T3 cells expressing a genetic construct encoding the estrogen-binding domain of the er fused to the c-Myc protein (myc–er cells) (6). The c-Myc function of this fusion is low in the absence of ligand but is activated when estradiol is added to growth-arrested myc–er cells. Expression of eIF-4E increased within 0.5–2 hr after addition of estradiol to myc–er cells and reached a maximum by 4–8 hr (Fig. 3). eIF-2α expression started to increase somewhat later and also reached its maximum 4–8 hr after induction. In contrast, addition of estradiol to control BALB/c 3T3 cells did not induce expression of eIF-4E or eIF-2α (Fig. 3, control lanes). Furthermore, neither elongation factor EF-1α nor translation initiation factor eIF-4A were induced in myc–er cells by estradiol (Fig. 3).

To determine whether c-*myc* stimulated transcription of eIF-4E and eIF-2α, we performed nuclear run-on and mRNA stability studies. Nuclear run-on studies demonstrated a marked increase in additional synthesis of eIF-4E and eIF-2α mRNAs 4 hr after estradiol addition (Fig. 4). Because eIF-4E and eIF-2α mRNA levels decayed at the same rate in cells treated with actinomycin D in the absence or presence of estradiol, estradiol stimulation of c-*myc* did not affect mRNA stability (Fig. 4). The transcriptional regulation of eIF-4E and eIF-2α we observed differs from the posttranscriptional regulation of eIF-2α previously described in T cells (11).

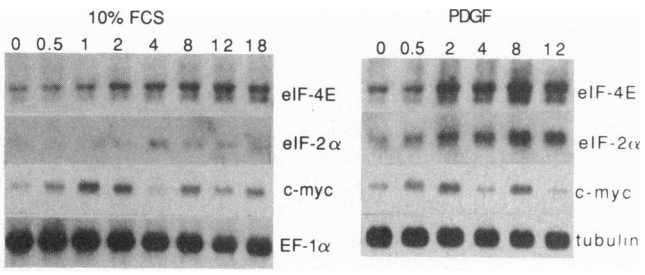

FIG. 1. Increases in expression of eIF-4E and eIF-2α follow increased c-*myc* expression in 3T3 cells stimulated by serum or PDGF. The growth of NIH 3T3 cells was arrested by incubation in medium/0.5% fetal bovine serum (FCS) for 3 days. The cells were subsequently stimulated by the addition of medium containing either 10% fetal bovine serum or PDGF at 50 ng/ml (GIBCO) for the hours indicated above each lane. Total cellular RNA was extracted at the indicated time points, and RNA blots were probed with the indicated ^{32}P-labeled fragments.

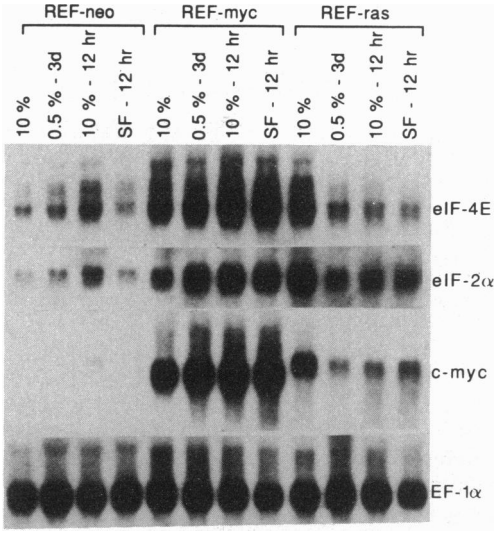

FIG. 2. Constitutive expression of c-Myc in transfected rat embryo fibroblasts induces parallel high levels of expression of translation initiation factors eIF-4E and eIF-2α. RNA blots were used to examine expression of 10 μg of RNA probed for translation initiation factor eIF-4E, eIF-2α, c-Myc, and elongation factor EF-1α. Gene expression was analyzed in REFs transfected with a plasmid encoding a G418-resistance marker alone (REF-neo) or cotransfected with an expression plasmid for c-*myc* (REF-myc) or *ras* (REF-ras) and the separate neomycin resistance-expressing plasmid. Gene expression was examined in these three types of cells maintained under four different conditions. We first compared continuously proliferating cells in 10% serum (10% lanes) to cells deprived of serum for 3 days (0.5% - 3d lanes); we further examined the expression of eIF-4E, eIF-2α, and c-Myc in cells stimulated by serum for 12 hr after serum deprivation (10% - 12 hr); and finally, we compared expression of eIF-4E, eIF-2α, and c-Myc in cells given fresh serum-free medium after serum deprivation (SF - 12 hr). As can be seen, the levels of eIF-4E and eIF-2α mRNA correlated with levels of c-Myc RNA in all cell lines under all conditions.

Because the activities of eIF-4E and eIF-2α are rate-limiting, both are critical elements in regulating protein synthesis (12). To assess the role of c-*myc* in activating protein synthesis, we measured amino acid incorporation in myc–er cells after estradiol addition (data not shown). A 60% increase in amino acid incorporation 4 hr after estradiol addition showed enhanced protein synthesis resulting from c-*myc* induction. Consistent with the earlier report (16), DNA synthesis measured by [^{3}H]thymidine incorporation started to increase in the myc–er cells 12 hr after estradiol addition.

To identify Myc-responsive sites in the eIF-2α gene, we examined the role of several candidate sites in the eIF-2α promoter (23). Footprinting and DNase hypersensitivity studies had previously identified potential regulatory elements at −550 to −600, −74 to −21, and +390 in the promoter and first intron of eIF-2α. In preliminary experiments, we deleted these elements in sequential chloramphenicol acetyltransferase (CAT) constructs and found that a construct containing only the isolated −74 to −21 nucleotide promoter element (PAL) was 4-fold more active in REF-myc cells than in REF-neo cells (data not shown). To identify sites specifically interacting with c-*myc* or a c-*myc*-induced protein complex, we performed EMSAs (Fig. 5). Substantially

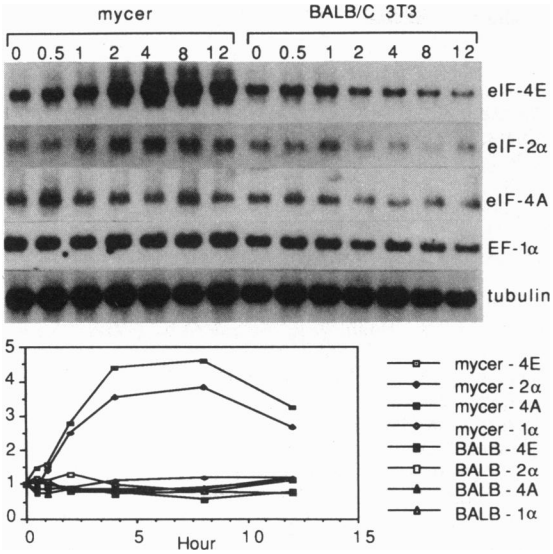

FIG. 3. Expression of translation initiation factors eIF-4E and eIF-2α is induced by addition of estradiol to cells transfected with a Myc–er fusion gene. Cells transfected by the Myc–er-encoding fusion gene (mycer) were made quiescent and then treated with 0.5 μM estradiol, and RNA was harvested at the indicated time points (0, 0.5, 1, 2, 4, 8, and 12 hr after estradiol addition). RNA blots (10 μg of total RNA per lane) were hybridized with ^{32}P-labeled cDNAs for eIF-4E, eIF-2α, eIF-4A, EF-1α, and α-tubulin to detect transcripts of these genes.

higher levels of PAL-binding activity were observed in REF-myc compared with REF-neo cells. Furthermore, extracts from two cell lines with amplified c-*myc* DNA sequences, HL-60 (Fig. 5) and COLO 320, contained the binding activity. In contrast, Hela cells that do not contain amplified c-*myc* sequences did not exhibit this complex (Fig. 5). Mutations in the core CGCATG motif of this PAL oligonucleotide (from CGCATG to CTCATC) abolished the binding activity. In contrast, binding to an E box oligonucleotide corresponding to the upstream region of the eIF-2α promoter (CACTTG) was equivalent in REF-neo and REF-myc cells.

DISCUSSION

Few previous reports have indicated whether factors regulating translation are transcriptionally regulated (11, 24). In this study, we demonstrated increased expression of translation initiation factors eIF-4E and eIF-2α after growth induction of fibroblasts. Maximum expression of eIF-4E and

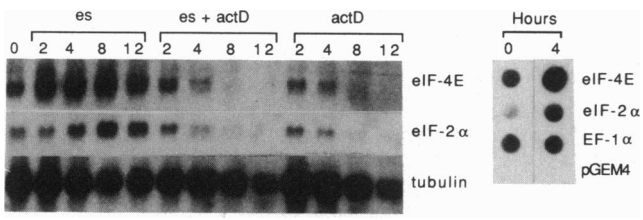

FIG. 4. Transcription of translation initiation factors eIF-4E and eIF-2α is induced by addition of estradiol to myc–er cells. (*Left*) Actinomycin D (5 μg/ml) was added to estradiol-stimulated myc–er cells in the indicated lanes. es, Estradiol alone; es + actD, both estradiol and actinomycin D were added simultaneously; actD, actinomycin D alone was added. Actinomycin D blocked increased expression of both genes (compare eIF-4E, eIF-2α, and α-tubulin levels at 2 and 4 hr versus 8 and 12 hr), and addition of estradiol had no effect on the stability of eIF-4E, eIF-2α, and α-tubulin mRNA (compare es + actD to actD alone). (*Right*) Nuclei from resting *myc–er* cells (column 0) or *myc–er* cells stimulated by estradiol (0.5 μM) for 4 hr (column 4) were harvested, and nuclear run-on assays were done as described.

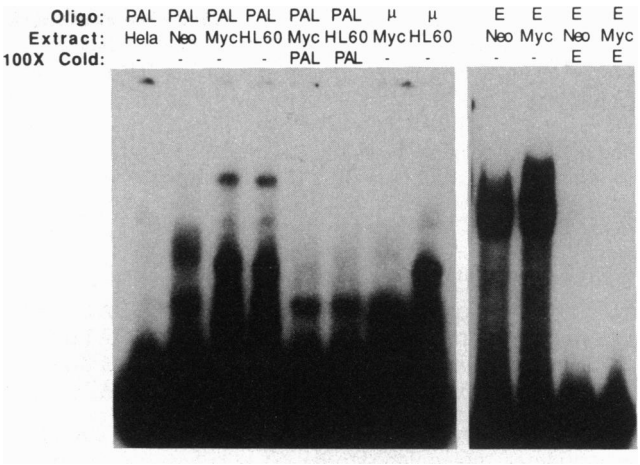

FIG. 5. Gel-retardation assays identify a sequence element in the translation initiation factor eIF-2α promoter for which DNA–protein interactions correlate with Myc expression. Nuclear extracts were prepared from 10^8 HeLa, REF-Neo, REF-myc, and HL-60 cells during logarithmic growth for EMSAs. Binding reactions included 5 μg of the indicated extracts. Neo, REF-neo; Myc, REF-myc or the indicated extracts plus a 100-fold excess of the indicated nonradioactive probes. We demonstrated PAL oligonucleotide binding in *myc*-expressing cells that was absent from REF-neo cells. This activity was seen in both *myc*-overexpressing cells (HL-60 and Ref-myc) but was absent from Ref-Neo and HeLa cells that did not overexpress *myc*. The absence of a shifted band in competition experiments with cold PAL and using a mutant PAL oligonucleotide (μ) demonstrated the specificity of the interacting sequence. [This *myc*-specific activity was seen when poly(dI·dC) was used as the nonspecific competitor DNA but was less clearly seen when using herring sperm DNA as the nonspecific competitor.] Finally, an E box oligonucleotide corresponding to the only E box motif identified in the eIF-2α promoter showed no difference in activity between Ref-neo and Ref-myc cells, confirming equivalent loading of protein in our extracts.

eIF-2α occurred at a time when peak expression of delayed early-growth genes has been demonstrated in fibroblasts (25). Increased expression of delayed-early-growth genes is thought to be the consequence of regulation by the immediate-early-growth response genes. Because c-*myc* is an important member of the immediate early group of genes (26) whose expression peaks at a time when new protein synthesis is critical to cell-cycle progression, a biological connection between c-*myc* and translation seemed plausible. Consequently, we examined translation initiation factors eIF-4E and eIF-2α in *myc*-transfected fibroblasts and in a cell line with inducible function of c-*myc* to show that these increases were from transcriptional increases occurring downstream of c-*myc* activation. However, further studies will be required to establish the mechanism of the interaction between c-*myc* and the promoter regions of the eIF-4E and eIF-2α genes.

Although the importance of c-*myc* in the neoplastic process is well recognized, few candidate *myc*-regulated genes have been identified, and the biological mechanism of c-*myc* action is poorly understood (5). Interestingly our data show that c-*myc* increases the expression of translation factors eIF-4E and eIF-2α, which are rate-limiting in protein synthesis. The critical functions of eIF-4E and eIF-2α in regulating rates of translation would be important in a broad range of tissue types, explaining why c-*myc* is implicated in growth control in many different tissues. In addition, translational control of gene expression may provide a potential element of specificity. Kozak (27) described a set of genes involved in growth regulation that have highly structured 5′ noncoding mRNA sequences that may confer dependence on higher levels of initiation factors to increase translational efficiency.

Our EMSA identified the PAL site in the eIF-2α promoter as a potential *myc*-regulated sequence. This site has also been identified as the nuclear respiratory factor 1 (NRF-1) sequence in the promoters of a group of enzymes that are rate-limiting in various metabolic pathways (28). The NRF-1 consensus sequence is intriguingly similar to the highest-affinity c-*myc*-binding sequence identified by *in vitro* studies (29):

Myc-binding site	CAC(G/A)TG
NRF 1 consensus	(T/C)CCGCA(T/C)GCGC(A/G)
eIF-2α translation initiation factor	TCCGCATGCGCG
tyrosine aminotransferase	TGCACATGCGCA
cytochrome *c* oxidase subunit Vb	CGCACATGCGCA
cytochrome *c* oxidase subunit Vlc-2	CACGCATGCGCA

A protein that binds the NRF-1 sequence can be purified as a peptide of molecular size 65–68 kDa (23, 28). Thus, our identification of c-*myc* interactions at the NRF-1-binding site suggests that NRF-1 (PAL) may be induced by c-*myc*, may associate with c-*myc*, or may be functionally similar to c-*myc*.

The role of oncogenes in regulating the rate of protein synthesis has received little attention. However, *ras* and *src* were previously shown to increase the phosphorylation and activity of eIF-4E (30, 31). Evidence that increased expression of eIF-4E and that inhibition of a suppressor of eIF-2α both cause neoplastic transformation clearly places these factors at an important control point in the cell cycle. Consequently, our finding that c-*myc* increased expression of eIF-4E and eIF-2α suggests that these translation initiation factors are important mediators of the biological functions of c-*myc*.

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