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Regulation of an Eukaryotic Initiation Factor-2 (eIF-2) Associated 67 kDa Glycoprotein (p^{67}) and Its Requirement in Protein Synthesis

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The p^{67} mRNA level and p^{67} requirement in protein synthesis were studied using an animal cell (KRC-7, rat tumor hepatoma cell) in culture. p^{67} mRNA was present in confluent cells but disappeared almost completely from serum-starved cells. However, when PMA was added to the serum-starved cells, p^{67} mRNA appeared in increasing quantities. Several-fold molar excess of p^{67} mRNA over that present in confluent cells was detected within 2 h of PMA addition and this level remained the same during the 4 h of the experiment. p^{67} requirement in protein synthesis was studied using a p^{67} antisense DNA construct under a metallothionein gene promoter. Expression of this antisense DNA in the presence of zinc in PMA-induced serum-starved cells completely inhibited induced appearance of p^{67} mRNA and subsequent protein synthesis. These results suggest that p^{67} is regulated at the mRNA level and also that this protein factor is essential for protein synthesis.

Eukaryotic initiation factor Gene transcription Antisense DNA

AN important regulatory mechanism in animal cells involves phosphorylation of the α-subunit of a key peptide chain initiation factor, eIF-2, by one or more eIF-2 kinases such as HRI (heme-regulated protein synthesis inhibitor) and PKR (double-stranded RNA-dependent eIF-2 α kinase). This inactivates eIF-2 activity and inhibits protein synthesis. It is generally believed that these eIF-2 kinases remain in inactive forms and are activated under certain physiological conditions. In reticulocyte lysates, one of these inhibitors, HRI, is activated during heme deficiency and the other inhibitor, PKR, is activated in presence of double-stranded RNA and ATP [for reviews see (10, 11,15)].

Gupta and coworkers have previously reported that animal cells, including reticulocyte lysates, contain a 67 kDa glycoprotein, p^{67} (2,5,6,17,18). p^{67} protects eIF-2 α-subunit from eIF-2 kinase(s)-catalyzed phosphorylation. This promotes protein synthesis in the presence of active eIF-2 kinase(s). These reports also indicated that animal cells, including reticulocyte lysates, contain at least one eIF-2 kinase in active form (17). However, this eIF-2 kinase cannot phosphorylate eIF-2 α-subunit because p^{67}, also present in these cells, protects eIF-2 α-subunit from eIF-2 kinase(s)-catalyzed phosphorylation (17).

An important characteristic of p^{67} is that this protein is easily degraded and is induced under

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different physiological conditions. The levels of this protein correlate directly with the protein synthesis activities of the cells (17) or of the cell-free extracts (2). For example, p^{67} is rapidly deglycosylated in heme-deficient reticulocyte lysate (2). This inactivates p^{67} and inhibits protein synthesis. Using an animal cell (KRC-7) in culture, it was observed that p^{67} was present at a high level in confluent cells but disappeared rapidly upon serum starvation. p^{67} again appeared in increasing quantities upon mitogen addition to the serum-starved cells. Protein synthesis activities of the confluent, serum-starved and mitogen-stimulated cells correlated with the p^{67} levels in the cells (17). These results suggested that p^{67} is a critical factor in protein synthesis. The precise mechanisms of regulation of p^{67} level as to whether this protein is regulated at the mRNA level and also posttranslationally by protein glycosylation are not known.

We have attempted to gain a better understanding of the mechanisms of regulation of p^{67} level in the cells and also the requirements of p^{67} in protein synthesis. Using a Northern blot procedure we analyzed the p^{67} mRNA in the cells under different growth conditions. We also used a p^{67}-antisense RNA to inhibit p^{67} mRNA and examine the requirement of p^{67} in protein synthesis.

MATERIALS AND METHODS

Cell Culture

The cloned cell line KRC-7, derived from Reuber H35 rat hepatoma cells, was kindly provided by John Koontz (University of Tennessee, Knoxville). KRC-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO/BRL), containing 5% (v/v) fetal calf serum and 5% (v/v) calf serum. The experimental procedures were essentially similar to those described by Trevillyan et al. (20). For analysis, 6×10^5 cells were seeded onto 100-mm tissue culture dishes containing 10 ml of medium. Serum-starved cells were prepared by washing cell monolayers with Hank's balanced solution followed by culturing in serum-free DMEM for an additional 3 days. A portion of these cells, after 1 day of serum starvation, was treated with $ZnSO_4$ (50 μM) and was further incubated in the serum-free medium for an additional 2 days. The serum-starved cells with and without zinc treatment were then stimulated by addition of 1.5 μM phorbol 12-myristate 13-acetate (PMA) (Sigma) for 2 h.

Determination of RNA Levels

Cells were harvested and total RNA was isolated using the guanidium isothiocyanate method (3). RNA concentrations were calculated from absorbance at 260 nm. p^{67} RNA levels were determined by using standard Northern blot technique: 20 μg of total RNA from each sample was denatured and subjected to electrophoresis in 1% agarose-formaldehyde denaturing gels. The gels were stained with ethidium bromide to verify that each lane contained similar amounts of undegraded RNA. RNA was transferred onto nylon membranes (Biotrace HP™) by vacuum blotting using 7.5 mM NaOH. Membranes were baked for 2 h at 80°C and were incubated in a prehybridizing solution containing 1 g nonfat dry milk, 200 μl 0.5 M EDTA, 6 g NaH_2PO_4, 7 g SDS in 100 ml solution, pH 7.2, for 2 h at 60°C. For hybridization, the membranes were further incubated overnight at 60°C in the same solution containing 50% formamide and radiolabeled p^{67} or G3PDH antisense RNA probes. Antisense probes (5×10^5 cpm/ml) (p^{67}, 1 kilobase; G3DPH, 316 base) were synthesised by in vitro transcription of pGEM-p^{67} (22) or pTRI-G3PDH-rat (Ambion), respectively, using [α-^{32}P]CTP and T7 RNA polymerase following the procedure described in Maxiscript™ kit (Ambion). Blots were washed successively, twice with $2 \times$ SSC for 15 min at room temperature, twice with $1 \times$ SSC and 1% SDS for 30 min at 65°C, and twice with $0.1 \times$ SSC for 30 min at room temperature. RNA levels were detected by autoradiography using Kodak X-Omat AR film at −70°C with intensifying screens.

Plasmids and Oligonucleotides

The pMT vector replicates episomally and was used to synthesize antisense RNA (22). This vector contains a mouse metallothionein I promoter. Cells harboring this vector are resistant to the antibiotic hygromycin as a result of the expression of the hyg B phosphotransferase gene present in the construct. The steps employed to prepare pMT-antisense p^{67} construct is shown diagrammetically in Fig. 1. A sequence complementary to p^{67} mRNA was placed under control of the inducible promoter to get an antisense p^{67} construct. Plasmid pGEM-p^{67} was prepared as described previously (22). The pMT vector was digested with *Not*I, filled in with T4 polymerase, and then digested with *Hind* III. The pGEM-p^{67} was digested with *Ava*I, filled in with T4 polymerase, and then digested with *Hind* III. The two linearized DNA fragments pMT (10.78 kb) and p^{67} cDNA (−10 to 1473 bp) were ligated together to form pMT-antisense p^{67} DNA construct. The construct was then transformed in competent cell line (DH5αF′) by the heat shock method.

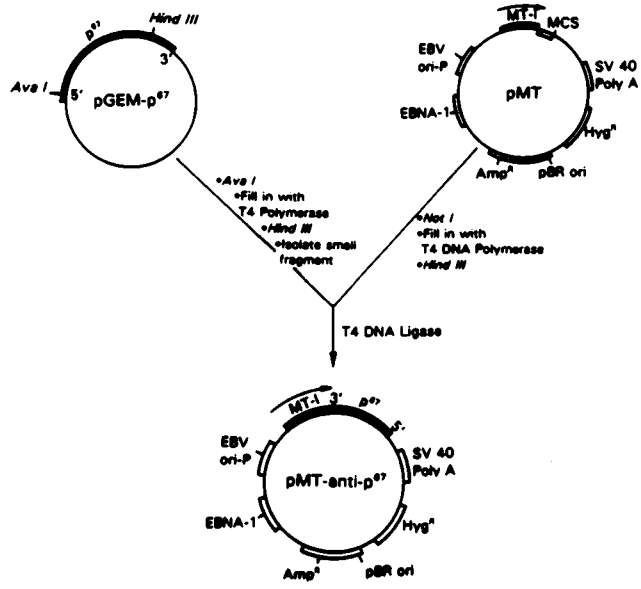

FIG. 1. Diagrammatic representation of the steps employed to construct the episomal vector pMT-antisense p^{67}. Abbreviations: EBNA-I, Epstein-Barr virus coded nuclear antigen I; EBV ori-P, Epstein-Barr virus origin of replication; MT-1, metallothionein-I promoter region; MCS, multiple cloning site; SV40 polyA, Simian virus 40 polyadenylation site; Hyg^R, hygromycin resistance gene (hyg B phosphotransferase); pBR ori, bacterial origin of replication; Amp^R, ampicillin resistance gene.

Characterization and Detection of the pMT-Antisense p^{67} DNA in KRC-7 Cells

The orientation of the p^{67} DNA insert was confirmed by using PCR and by DNA sequencing (data not shown). The existence of pMT-antisense p^{67} DNA in KRC-7 cells was confirmed by Southern blot of *Hind* III-digested episomal DNA (purified by Hirt extraction protocol) (12). The probe used was random primer labeled 300 bp from p^{67} cDNA (400–700 bp). A single band at the position 12.26 kb confirmed the presence of pMT-antisense p^{67} plasmid (data not shown). This result indicates that pMT-antisense p^{67} vector DNA was maintained as an episome inside the cells. In each case, the plasmids were isolated from 2×10^7 cells. The plasmids were *Hind* III digested and then quantitated by using the random primer labeled probe containing the p^{67} cDNA sequence (300 bp; 400–700 bp from p^{67} cDNA). Based on a molecular weight of 8,091,600 of pMT-antisense p^{67} construct, it was calculated that there were approximately 374 copy Nos./cell.

Transfection of the pMT-Antisense p^{67} DNA in KRC-7 Cell Line

The pMT constructs with and without p^{67} antisense DNA were used to transfect rat hepatoma cell line KRC-7 using lipofectin reagent (GIBCO/BRL). The cells were cultured in the complete DMEM to 50–70% confluency. The cells were grown for 18 h in the serum-free medium along with 50 μl lipofectin reagent and 5 μg of the construct. After transfection, the cells were grown in the complete DMEM for 2 days to get hygromycin-resistant phenotypes. The transformed cells were then selected in the complete medium with 0.2–0.4 mg/ml of hygromycin for 3 weeks. Only the cells containing the construct survived in this medium.

Immunologic Analysis

Immunoblot analysis was used to measure p^{67} protein levels in the wild-type cells and also in the transformed cells under different growth conditions. The wild-type KRC-7 cells and also the KRC-7 cells transformed with pMT vectors with and without antisense p^{67} DNA were grown to 50–70% confluency. The cells were then starved in serum-free medium for 3 days. On the second day of serum starvation, a portion of the cells was treated with $ZnSO_4$ (50 μM) and was further incubated in the serum-free medium for an additional 2 days. After 3 days of serum starvation, the cells, with and without zinc treatment, were incubated

with PMA (1.5 μM) for 2 h. Cell extracts were prepared as described (17). Equal amounts of proteins (100 μg) in each cell extract were used. The proteins were separated by SDS-PAGE using 15% gels. The proteins were transferred to Biotrace NT membranes and were immunoblotted using p^{67} monoclonal antibodies following the procedure as described previously (17).

Measurement of Rate of Protein Synthesis

Cells were grown in complete DMEM to 50–70% confluency (1×10^6) and were then starved in serum-free medium for 3 days. On the second day of serum starvation, a portion of the cells was treated with $ZnSO_4$ (50 μM) and was further incubated in the serum-free medium for an additional 2 days. After 3 days of serum starvation, the cells, with or without zinc treatment, were washed twice with phosphate-buffered saline (PBS) (GIBCO/BRL) and were incubated in short-term labeling medium [serum-free DMEM (GIBCO/BRL) lacking methionine] at 37°C with 10% CO_2 in an incubator for 15 min. The cells were then washed and incubated in short-term labeling medium for 30 min in the presence of 0.1 mCi/ml of [^{35}S]methionine. The cells were lysed in lysis buffer (17). [^{35}S]Methionine-labeled cell extracts (10 μg) were TCA precipated and the precipitates were filtered through Whatman GF/C filter discs (2.5 cm diameter). Radioactivity of the TCA precipitates was measured using a liquid scintillation counter.

RESULTS

p^{67} mRNA Levels

We determined p^{67} mRNA levels in KRC-7 cells during confluency, serum starvation, and mitogen stimulation of serum-starved cells using a standard Northern blotting procedure. The results are shown in Fig. 2. Equal amounts of RNA (20 μg) used in each experiment showed similar 28S rRNA bands when analyzed by gel electrophoresis followed by staining using ethidium bromide (data not shown). As shown in Fig. 2A, p^{67} mRNA was present in confluent cells (lane 1), and this mRNA almost completely disappeared during serum starvation for 3 days (lane 2). However, when the same serum-starved cells were treated with the mitogen PMA, p^{67} mRNA appeared within 30 min of mitogen addition (lane 3) and the level increased rapidly with time (lanes 4–7). Several-fold molar excess of p^{67} mRNA over that observed in confluent cells (lane 1) appeared within 2 h of mitogen addition (lane 6), and this level remained the same over the 4-h period (lane 7) used in this experiment. Under identical conditions, the levels of a control RNA such as G3PDH RNA remained essentially constant.

These results provide evidence that p^{67} mRNA level changes significantly during serum starvation and subsequent mitogen addition. This mRNA becomes undetectable during serum starvation and its appearance is enhanced in response to the mitogen, namely PMA.

Growth Characteristics of Wild-Type and Transformed KRC-7 Cells

To determine the viability of the KRC-7 cells during the experimental period, we analyzed the growth characteristics of these cells under different conditions (Fig. 3). In the enriched medium, the wild-type KRC-7 cells doubled in approximately 24 h (Fig. 3A), and addition of zinc did not have any significant effect on cell growth (Fig. 3A). In both the cases, the cell number remained the same during subsequent incubation in serum-free medium for 5 days. On the other hand, the KRC-7 cells transformed with pMT-antisense p^{67} vector grew considerably more slowly in the enriched medium with a doubling time of about 70 h (Fig. 3B). In the absense of zinc, the number of transformed cells remained the same during subsequent incubation in the serum-free medium for 4 days and thereafter showed some decline (Fig. 3B). In the presence of zinc, the number of transformed cells remained the same during subsequent incubation in the serum-free medium for 3 days and thereafter declined sharply (Fig. 3B). Zinc had no detectable effect on KRC-7 cells transformed with only pMT vector without the insert (data not shown).

The results show that the zinc-treated cells, transformed with pMT-antisense p^{67}, died after 3 days of serum starvation. In our experiments, we collected only the viable cells within 3 days of serum starvation.

Effects of Expression of Antisense p^{67} DNA on p^{67} mRNA Synthesis

We studied the effects of expression of p^{67} antisense DNA on p^{67} mRNA synthesis in KRC-7 cells during serum starvation and subsequently during PMA addition to the serum-starved cells. The results are shown in Fig. 4. p^{67} mRNA was undetectable in serum-starved cells (lane 1), but became detectable within 2 h of PMA addition (lane 2).

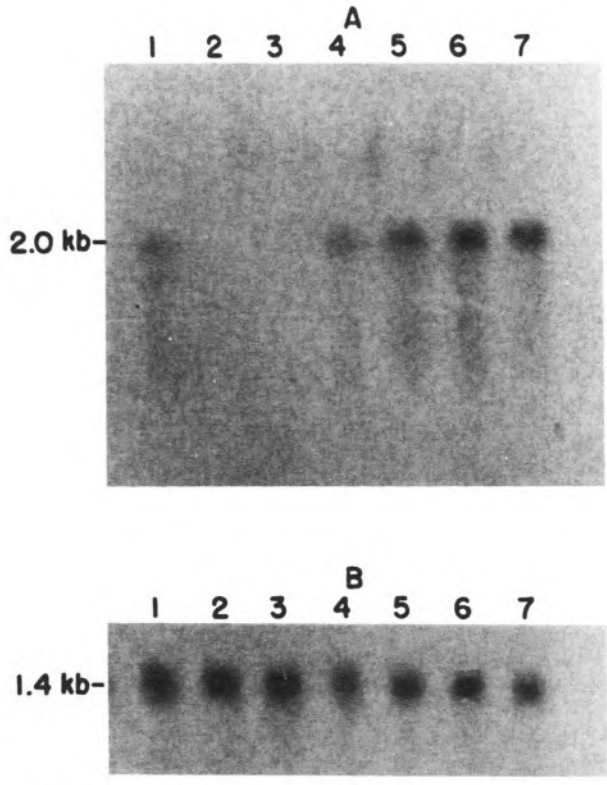

FIG. 2. Northern blot analysis of p^{67} mRNA and G3PDH mRNA in tumor hepatoma cells (KRC-7) that were confluent (50–70%), serum starved or PMA induced. Cells grown to 50–70% (lane 1) were serum starved for 72 h (lane 2). The starved cells were stimulated with PMA for different periods: lane 3, 15 min; lane 4, 30 min; lane 5, 1 h; lane 6, 2 h; lane 7, 4 h. RNA (20 μg) from different cells was analyzed by Northern blot analysis using antisense p^{67} RNA (A) and antisense G3PDH RNA probe (B).

Addition of zinc to either serum-starved cells (lane 3) or PMA-induced cells (lane 4) showed similar results to those observed in the absence of zinc. Identical experiments were done by using KRC-7 cells containing pMT-0 construct (only the pMT vector without the insert) (lane 5–8) or pMT-antisense p^{67} construct (lane 9–12). When KRC-7 cells transformed with pMT-0 construct were used, no difference was seen compared to the cells without vector (lane 5–8). KRC-7 cells transformed with pMT-antisense p^{67} were used in lanes 9–12. The experiments displayed in lanes 9 and 10 were done in the absence of zinc and the results were the same as those displayed in lanes 1 and 2. Some inhibition of p^{67} mRNA appearance was observed in the PMT-antisense p^{67} transfected cells after PMA addition (lane 10). This may be due to a low-level constitutive antisense p^{67} expression because of the presence of trace amount of zinc in the cell system. When zinc was added to the cells containing PMT-antisense p^{67}, p^{67} mRNA was undetected in the serum-starved cells (lane 11) and also in the PMA-induced serum-starved cells (lane 12). In identical sets of experiments, we measured G3PDH mRNA levels. The G3PDH mRNA levels did not change significantly over the period tested. To examine equal loading of the RNA samples, the gels were also stained with ethidium bromide (data not shown).

Immunoblot Analysis of p^{67}

Previously it was reported that p^{67} was undetectable in serum-starved cells and its synthesis could be induced by PMA addition to the serum-starved cells (17). The results presented in Fig. 5 show that this induced synthesis of p^{67} in the presence of PMA can be completely blocked by expression of pMT-antisense p^{67} DNA. Standard immunoblot analysis was performed to measure p^{67} level in the cells. The experiments displayed in Fig. 5 (lanes 1–4) were performed using wild-type se-

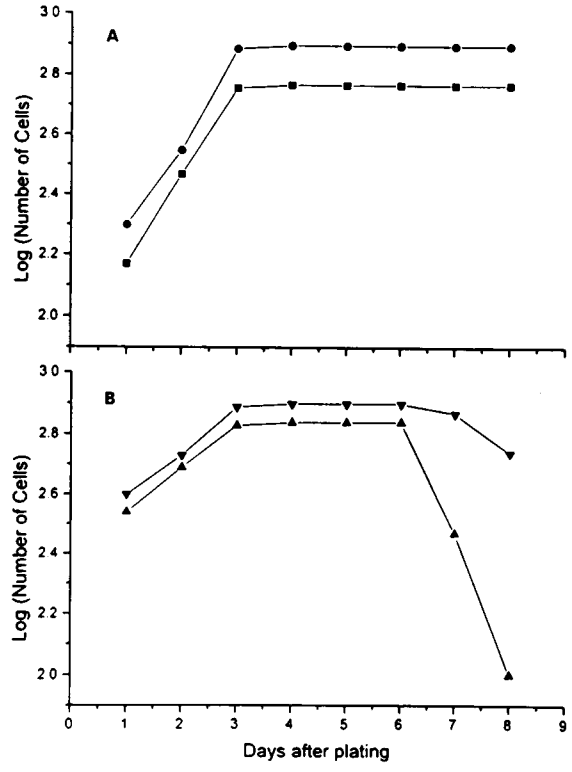

FIG. 3. Growth curves of KRC-7 cells and transformed cells. Cells were plated in 25-cm^2 flasks with a graduated bottom. The average cell number in four random 1-cm^2 grids was taken each day, beginning 1 day after plating. The cells were grown for 3 days in complete medium and were incubated in serum-free media. After the second day of incubation in the serum-free medium, a portion of the cells was treated with $ZnSO_4$ (50 μM). (A) KRC-7 cells: without (●), and with (■) the zinc. (B) Cells transfected with PMT-antisense p^{67} cultured in 0.3 mg/ml of hygromycin, without (▼) and with (▲) zinc.

rum-starved KRC-7 cells. p^{67} was absent in serum-starved cells (lane 1), but appeared in significant amounts within 2 h of PMA addition (lane 2). Addition of zinc to either serum-starved cells (lane 3) or PMA-induced cells (lane 4) had no effect on p^{67} synthesis. Similar sets of experiments as displayed in lanes 1–4 were performed by using KRC-7 cells containing either pMT-0 construct (lanes 5–8) or pMT-p^{67} antisense DNA construct (lanes 9–12). When cells transfected with pMT-0 construct were used (lanes 5–8), p^{67} level was absent in the serum-starved cells (lane 5) and its synthesis was similarly induced by PMA (lane 6) as in nontransformed cells. Also, addition of zinc had no effect on p^{67} synthesis both in the absence (lane 7) or presence of PMA (lane 8). The experiments displayed in lanes 9–12 were performed using pMT-antisense p^{67} DNA construct. As shown in lanes 1–4 and lanes 5–8, p^{67} was absent in serum-starved cells (lane 9) and its synthesis was induced in the presence of PMA (lane 10). However, some inhibition of p^{67} synthesis was observed (compare lanes 2 and 10). As mentioned earlier, this may be due to a low-level constitutive antisense p^{67} expression because of the presence of trace amount of zinc in the cell. These experiments were done in the absence of zinc. However, when zinc was added to the cells, p^{67} appearance was inhibited both in the serum-starved (lane 11) and in the PMA-induced cells (lane 12). Antisense-p^{67} DNA expression in the presence of zinc completely inhibited the induction p^{67} synthesis in the presence of PMA.

eIF-2 α-Phosphorylation

p^{67} protects eIF-2 α-subunit from eIF-2 kinase(s)-catalyzed phosphorylation and, therefore, loss of p^{67} is expected to increase eIF-2 α-subunit phosphorylation in the cells. To correlate p^{67} level in the cells and eIF-2 α-subunit phosphorylation, we analyzed endogenous eIF-2 α-subunit phosphorylation in KRC-7 cell extracts grown under different conditions. The results are shown in Fig. 6. The experiments displayed in Fig. 6 (lanes 1–4) were performed using wild-type serum-starved KRC-7 cells. Significant eIF-2 α-subunit phosphorylation was observed when the extract from quiscent cells was used (lane 1). This phosphorylation was completely inhibited after PMA addition to the quiscent cells (lane 2). Addition of zinc to either serum-starved cells (lane 3) or PMA-induced cells (lane 4) had no effect on eIF-2 α-subunit phosphorylation. Similar sets of experiments as displayed in lanes 1–4 were performed by using KRC-7 cells containing either pMT-0 construct (lanes 5–8) or pMT-p^{67} antisense DNA construct (lanes 9–12). When cells transfected with pMT-0 construct were used (lanes 5–8), eIF-2 α-subunit phosphorylation was increased in the serum-starved cells (lane 5) and it decreased on PMA addition (lane 6) as in nontransformed cells. Also, addition of zinc had no effect on eIF-2 α-subunit phosphorylation, both in the absence (lane 7) or presence of PMA (lane 8). The experiments displayed in lanes 9–12 were performed using the cells transfected with pMT-antisense p^{67} DNA construct. As shown in lanes 1–4 and lanes 5–8, eIF-2 α-subunit phosphorylation was increased in serum-starved cells (lane 9) and it decreased in the presence of PMA (lane 10). These experiments were done in the absence of zinc. However, when zinc was added to the cells, eIF-2 α-subunit phosphorylation was increased in serum-starved cells (lane 11) and also in serum-

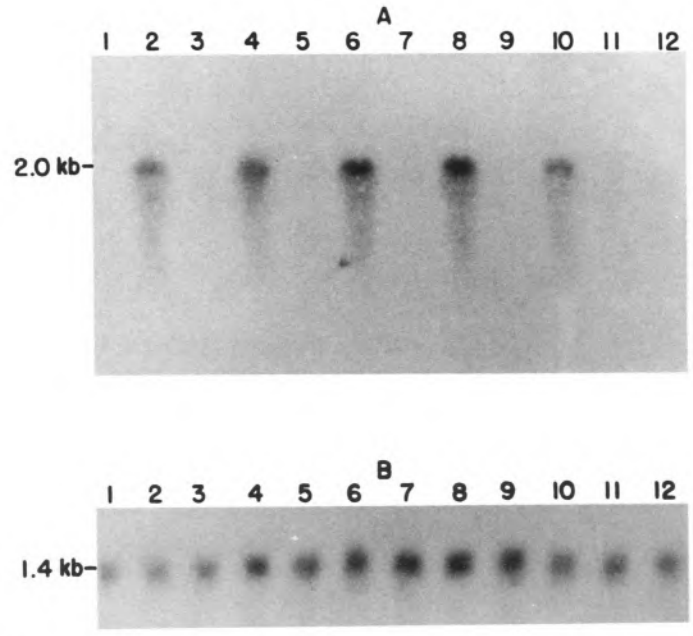

FIG. 4. Northern blot analysis of p^{67} mRNA and G3PDH mRNA in wild-type KRC-7 cells and in the transformed KRC-7 cells (transformed with pMT-0 and pMT-antisense p^{67} construct) that were serum starved, zinc treated, or PMA induced. Confluent KRC-7 cells (50–70%) were serum starved (A, lane 1) and then were stimulated with PMA (A, lane 2). Serum-starved KRC-7 cells were also treated with zinc sulphate (A, lane 3) and subsequently stimulated with PMA (A, lane 4). Similar sets of experiments were done with KRC-7 cells transformed with pMT-0 (A, lanes 5–8) or pMT-antisense p^{67} (A, lanes 9–12). As control, G3PDH mRNA content was measured using similar sets of cells as the above experiment (B). RNA (20 μg) was used in for Northern blotting.

starved cells even in the presence of PMA (lane 12).

Measurement of the Rate of Protein Synthesis

We determined the protein synthesis activities of the wild-type and transformed cells under different growth conditions. We measured [^{35}S]methionine incorporation into proteins in intact cells during 30-min incubation following the procedure described previously (6). The results are shown in Table 1. No significant difference in protein synthesis was observed in serum-starved cells with and without pMT (pMT-0 or pMT-antisense p^{67})

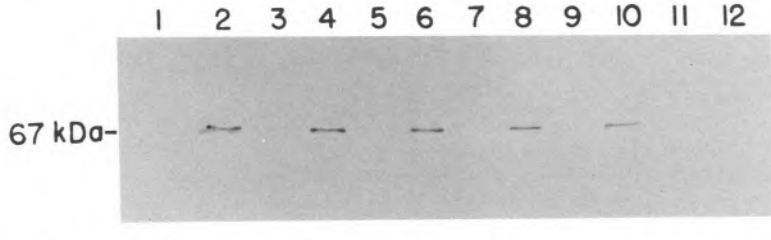

FIG. 5. Immunoblot analysis of p^{67} in wild-type KRC-7 and the transformed KRC-7 cells (transformed with pMT-0 and pMT-antisense p^{67}) that were serum starved, zinc treated, or PMA induced. Confluent KRC-7 cells (50–70%) were serum starved (lane 1) and then were stimulated with PMA (lane 2). Serum-starved KRC-7 cells were also treated with zinc sulphate (lanes 3) and subsequently stimulated with PMA (lane 4). Similar sets of experiments were done with KRC-7 cells transformed with pMT-0 (lanes 5–8) or pMT-antisense p^{67} (lanes 9–12). Approximately 100 μg protein in each cell lysate was used for immunoblotting using p^{67} monoclonal antibodies. Detailed immunoblot procedure for analysis of p^{67} is described under Materials and Methods.

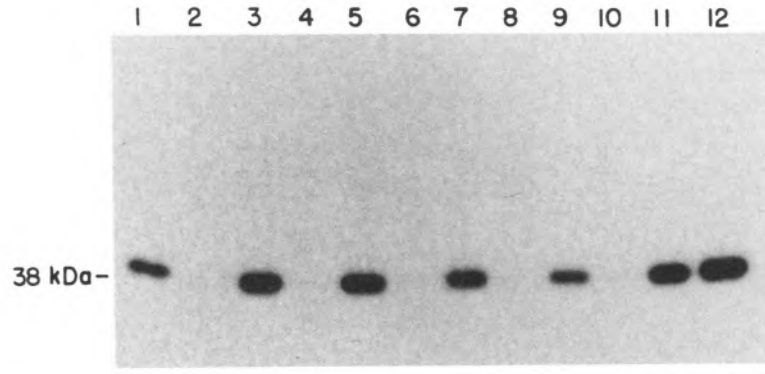

FIG. 6. Determination of eIF-2 α-subunit phosphorylation in in wild-type KRC-7 and the transformed KRC-7 cells (transformed with pMT-0 and pMT-antisense p^{67}) that were serum starved, zinc treated, or PMA induced. Reaction conditions for eIF-2 α-subunit phosphorylation were the same as described by Datta et al. (5). Reaction mixtures (25 μl) contained 20 mM Tris-HCl (pH 7.8), 100 mM KCl, 10 μg of BSA, 2 mM dithiothreitol, 1 mM Mg^{2+}, 40 μM ATP, and 60 μg different cell lysates. The reaction mixtures were mixed with 10 μCi [α-^{32}P]ATP and were incubated at 37°C for 10 min. The eIF-2 α-subunit in the reaction mixtures was immunoprecipitated using eIF-2 α polyclonal antibodies and protein A agarose. The immunoprecipitates were subsequently analyzed by SDS-PAGE followed by autoradiography. A detailed description of the experiments is given in the text.

constructs. However, expression of antisense p^{67} DNA in the presence of zinc led to a sixfold decrease in methionine incorporation (38×10^3 cpm to 6.0×10^3 cpm). Under identical conditions, and in the presence of zinc, no significant decrease in protein synthesis was observed in cells transformed with pMT-0 (38×10^3 cpm to 31×10^3 cpm). Addition of PMA to the serum-starved cells increased protein synthesis by approximately 2.5-fold (32×10^3 cpm to 76×10^3 cpm). This activity remained essentially the same in the pMT-0 transformed cells. Also, addition of zinc did not have any significant effect on the protein synthesis activity. However, expression of pMT-antisense p^{67} DNA in the presence of zinc almost completely inhibited this PMA induction of protein synthesis (78×10^3 cpm to 8×10^3 cpm).

DISCUSSION

Our previous reports indicated that p^{67} level in the KRC-7 cells changed significantly under different growth conditions. p^{67} was present at a high level in confluent cells, became undetectable upon serum starvation, and reappeared quickly after PMA addition to the serum-starved cells (17). Our previous results indicated p^{67} activity in the cells is regulated by protein glycosylation–deglycosylation (2). The results presented in this article provide evidence that p^{67} synthesis is also regulated at the mRNA level. p^{67} mRNA was present in the confluent cells and this mRNA was undetectable upon serum starvation. Again, p^{67} mRNA appeared in increasing quantities upon PMA addition to the serum-starved cells. Significant p^{67}

TABLE 1
MEASUREMENT OF PROTEIN SYNTHESIS IN SERUM-STARVED KRC-7 CELLS AND TRANSFORMED KRC-7 CELLS

Addition	[^{35}S]Methionine Incorporated (cpm x 10^3)		
	Serum-Starved KRC-7 Cells	Transformed KRC-7 Cells (+ pMT-0)	Transformed KRC-7 Cells (+ pMT-Antisense p^{67})
None	32	38	26
Zn	38	31	6.0
PMA	76	75	56
PMA + Zn	78	73	8.0

The experimental procedures were the same as described in the Materials and Methods section.

mRNA was detected within 30 min of PMA addition and this mRNA level increased with time. Several-fold molar excess of p^{67} mRNA over and above that present in the confluent cells was detected within 2 h of PMA addition. In this respect, p^{67} appeared similar to several "Immediate Early Genes" such as *fos* and *jun* whose transcriptions are significantly enhanced after mitogen addition to the serum-starved cells (4,9,14,16,19). However, unlike *fos* and *jun* transcription, p^{67} mRNA transcript was stable during the 4-h period used in the present experiment.

In this study we expressed an antisense-p^{67} DNA construct and analyzed the effects of expression of this DNA on p^{67} mRNA and p^{67} protein synthesis and also on overall protein synthesis in the cells. Some significant observations are noted.

Effects of Antisense-p^{67} DNA Expression on p^{67} mRNA and p^{67} Protein Synthesis

Expression of antisense-p^{67} DNA led to an almost complete disappearance of p^{67} mRNA upon mitogen addition to the serum-starved cells (Fig. 4). It should be pointed out that although antisense RNA technology has been used in several cases to inhibit expression of a specific mRNA, the mechanisms of inhibition may vary. The antisense RNA is expected to form RNA duplex with the mRNA and thereby either inhibits processing of mRNA and transport of the mRNA from the nucleus, prevents its translation, or enhances degradation of mRNA (8). There are examples for each cases [see (1)]. Using a pMT vector, Trojan et al. (21) reported almost complete disappearance of IGF-I mRNA upon synthesis of antisense IGF-I transcript. Similarly, expression of eIF-4E antisense RNA significantly reduced eIF-4E mRNA in transfected cells (1). We also observed complete disappearance of p^{67} mRNA upon induction of p^{67} antisense transcripts (Fig. 6). Our results thus suggest that the RNA duplex formed between p^{67} mRNA and p^{67} antisense RNA is unstable. As expected, loss of p^{67} mRNA upon expression of antisense-p^{67} DNA led to an almost complete loss of p^{67} protein synthesis (Fig. 5).

Effects of Antisense-p^{67} DNA Expression on Overall Protein Synthesis

As shown in Table 1, the expression of antisense-p^{67} DNA resulted in a sixfold decrease in protein synthesis in serum-starved cells. Addition of PMA to the serum-starved cells increased protein synthesis by 2.5-fold. Again, expression of antisense-p^{67} RNA almost totally suppressed PMA induction of protein synthesis; an almost 10-fold decrease in protein synthesis was observed. These results provide evidence that p^{67} is essential for protein synthesis. It should be noted that protein synthesis in the serum-starved cells increased only by 2.5-fold (32×10^3 cpm to 76×10^3 cpm) upon PMA addition. Under similar conditions, p^{67} mRNA level was increased many fold (Fig. 4). We offer the following explanation. p^{67} protein and p^{67} mRNA are present in the serum-starved cells at a very low and undetectable level. This low steady-state level of both p^{67} protein and p^{67} mRNA is determined by continuous synthesis and degradation of p^{67} protein and p^{67} mRNA. Inhibition of appearance of new p^{67} mRNA by p^{67} antisense RNA eliminates this low level of endogeneous p^{67} mRNA. This inhibits synthesis of p^{67} protein. We believe p^{67} is necessary at a catalytic level for protein synthesis and the low level of p^{67} in the serum-starved cells is sufficient to promote protein synthesis at a reasonable rate, as observed in the experiment described in Table 1. Upon PMA addition, both p^{67} mRNA (Figs. 2 and 4) and p^{67} protein synthesis (Fig. 5) were increased. However, under these conditions, p^{67} protein reached a saturating level for protein synthesis and other factors in protein synthesis became limiting. Thus, although the levels of both p^{67} protein and p^{67} mRNA were significantly increased after PMA addition, this effect was not similarly reflected in overall protein synthesis. Expression of antisense-p^{67} DNA almost completely eliminated endogenous p^{67} mRNA and inhibited low level of p^{67} synthesis. This led to an almost 10-fold decrease in protein synthesis. These results thus provide a more convincing argument that p^{67} is necessary for overall protein synthesis.

It should be noted that despite numerous reports over the past two decades, we have not yet gained a clear understanding of the mechanism of mammalian peptide chain initiation and identified all the necessary protein factors for peptide chain initiation. There are conflicting reports regarding the mechanisms of in vitro assemblies of different preinitiation complexes and the protein factors involved in the process. Several alternative approaches have been attempted to demonstrate the requirement of individual protein factor(s) in protein synthesis. Recently Benedetti et al. (1) reported that expression of antisense RNA against initiation factor eIF-4E mRNA led to inhibition of protein synthesis. This result suggested that eIF-4E is an essential component of protein synthesis. In this research, we applied a similar procedure using an antisense RNA against p^{67}. Expres-

sion of the antisense-p^{67} RNA strongly inhibited protein synthesis in transformed cells. These results thus provide an additional evidence that p^{67} is an essential factor for protein synthesis.

Our results showed that expression of antisense-p^{67} cDNA led to loss of p^{67} mRNA (Fig. 4) and p^{67} protein (Fig. 5). As expected, loss of p^{67} protein accompanied increased eIF-2 α-subunit phosphorylation (Fig. 6) and inhibition of protein synthesis (Table 1). It should be noted that expression of antisense-p^{67} cDNA is expected to form a duplex RNA between p^{67} mRNA and antisense-p^{67} RNA. However, previous reports indicated that this duplex RNA is formed in the nucleus and is rapidly degraded in the nucleus (13,23). Possibilities exist that RNA duplex, if present in cytoplasm, could inhibit protein synthesis by activation of an eIF-2 kinase, PKR. Because this duplex was not detected in the cytoplasm under our experimental conditions, this possibility may be excluded.

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REFERENCES

1. Benedetti, A. D.; Joshi-Barve, S.; Rinker-Schaeffer, C.; Rhoads, R. E. Mol. Cell. Biol. 11: 5435–5445; 1991.
2. Chakraborty, A.; Saha, D.; Bose, A.; Chatterjee, M.; Gupta, N. K. Biochemistry 33:6700–6706; 1994.
3. Chomczynnski, P.; Sacchi, N. Anal. Biochem. 162: 156–159; 1987.
4. Curran, T.; Franza, B. R., Jr. Cell 55:395–397; 1988.
5. Datta, B.; Ray, M. K.; Chakrabarti, D.; Gupta, N. K. Indian J. Biochem. Biophys. 25:478–482; 1988.
6. Datta, B.; Chakrabarti, D.; Roy, A. L.; Gupta, N. K. Proc. Natl. Acad. Sci. USA 85:3324–3328; 1988.
7. Datta, B.; Ray, M. K.; Chakrabarti, D.; Wylie, D.; Gupta, N. K. J. Biol. Chem. 264:20620–20624; 1989.
8. Green, P. J.; Pines, O.; Inouye, M. Ann. Rev. Biochem. 55:569–597; 1986.
9. Greenberg, M. E.; Ziff, E. B. Nature 311:433–438; 1984.
10. Gupta, N. K.; Datta, D. Ray, M. K.; Roy, A. L. In: Ilan, J., ed. Translational regulation in gene expression. New York: Plenum Publishing Corp.; 1992: 287–334.
11. Hershey, J. W. B. Annu. Rev. Biochem. 60:717–755; 1991.
12. Hirt, B. J. Mol. Biol. 26:365–369; 1967.
13. Kim, S. K.; Wold, B. J. Cell 42:129–138; 1985.
14. Lamph, W. W.; Wamsley, P.; Sassone-Corsi, P.; Verma, I. M. Nature 334:629–631; 1988.
15. London, I, M.; Levin, D. H.; Matts, R. L.; Thomas, N. S. B.; Petryshyn, R.; Chen, J. J. In: Boyer, P. D.; Krebs, E. G., eds. Enzymes 3rd ed., vol. XVII. City: Publisher; 1987:359–380.
16. Quantin, B.; Breathnach, R. Nature 334:538–539; 1988.
17. Ray, M. K.; Datta, B.; Chakrabarty, A.; Chattopadhyay, A.; Meza-Keuthen, S.; Gupta, N. K. Proc. Natl. Acad. Sci. USA 89:539–543; 1992.
18. Ray, M. K.; Chakraborty, A.; Datta, B.; Chattopadhyay, A.; Saha, D.; Bose, A; Kinzy, T.; Wu, S.; Hileman, R. E.; Merrick, W. C.; Gupta, N. K. Biochemistry 32:5151–5159; 1993.
19. Ryseck, R. P.; Hirai, S. I.; Yaniv, M.; Bravo, R. Nature 334:629–631; 1988.
20. Trevillyan, J. M.; Kulkarni, R. K.; Byers, C. V. J. Biol. Chem. 259:897–902; 1984.
21. Trozan, J.; Blossey, B. K.; Johnson, T. R.; Rudin, S. D.; Tykocinski, M.; Ilan, J.; Ilan, J. Proc. Natl. Acad. Sci. USA 89:4874–4878; 1992.
22. Wu, S.; Gupta, S.; Chatterjee, N.; Hileman, R. E.; Kinzy, T. G.; Denslow, N. D.; Merrick, W. C.; Chakrabarty, D.; Osterman, J. C.; Gupta, N. K. J. Biol. Chem. 268:10796–10801; 1993.
23. Yokoyama, K.; Imamoto, F. Proc. Natl. Acad. Sci. USA 84:7363–7364; 1987.