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78-Kilodalton Glucose-Regulated Protein Is Induced in Rous Sarcoma Virus-Transformed Cells Independently of Glucose Deprivation

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To identify mRNAs with altered expression in Rous sarcoma virus (RSV)-transformed cells, we screened a chicken embryo fibroblast (CEF) cDNA library by differential hybridization. One clone, designated R1H, showed markedly elevated mRNA expression in RSV-transformed cells. Nucleotide sequence analysis indicated that R1H mRNA encodes 78-kilodalton glucose-regulated protein (GRP78). Chicken GRP78 was found to be very highly conserved in comparison with rat GRP78 (96% identity between chicken and rat amino acid sequences). In contrast to previous observations, we found that GRP78 was induced in RSV-transformed cells in the absence of glucose deprivation. When cells were grown in glucose-supplemented medium, the level of GRP78 mRNA was approximately fivefold higher in RSV-transformed CEF than in transformation-defective virus-infected or uninfected CEF. Similar changes in GRP78 protein content were also found. Using a temperature-sensitive mutant of RSV and supplemental glucose, we found a gradual increase in the level of GRP78 mRNA beginning at 4 h after shiftdown to permissive temperature. Uridine supplementation did not block the induction seen in CEF infected with a temperature-sensitive mutant. These results indicate that GRP78 is induced by $p60^{v\text{-}src}$ in the absence of glucose deprivation.

One approach to understanding how oncogenes induce neoplasia is to identify genes with altered expression in transformed cells. Changes in the expression of cellular genes may be relevant to phenotypic features of the transformed cell, such as uncontrolled growth, invasiveness, resistance to immune rejection, and metastatic spread. In addition, studying the signals that control the expression of such genes can provide insight into the mechanisms of action of oncogenes.

We have been investigating the changes in cellular gene expression that occur in chicken embryo fibroblasts (CEF) after transformation by Rous sarcoma virus (RSV). In the initial stage in this project, we have isolated clones from CEF cDNA or RSV-transformed CEF cDNA libraries that are expressed at different levels in RSV-transformed cells compared with uninfected cells (38). It is likely that many of the mRNAs identified by this approach are regulated in normal cell growth. Altered expression of such mRNAs in RSV-transformed CEF may contribute to or result from altered growth processes after transformation. One such gene, designated 9E3, was found to encode a protein similar to human connective tissue-activating peptide (1, 38). In this paper we describe the isolation and characterization of another cDNA and report on the expression of this gene in RSV-transformed and normal cells.

MATERIALS AND METHODS

Cell cultures and viruses. Secondary CEF were maintained in Ham F10 medium supplemented with 5% calf serum, 1% chicken serum, 10% tryptose pentose broth, 100 U of penicillin per ml, 50 μg of streptomycin per ml, and 2 μg of amphotericin B per ml. The medium was changed every 24 h unless otherwise noted. In some experiments, glucose was added to achieve a final concentration of 24 mM (4.32 g/liter). To assay the effects of uridine supplementation, the medium was changed to RPMI-1640 medium (GIBCO Laboratories) supplemented with 5% dialyzed calf serum (GIBCO Laboratories), 5 mM uridine, and antibiotics. Virus infection was done as described elsewhere with Schmidt-Ruppin A strain of RSV for wild-type infection; a transformation-defective virus, *td*107, as a control for the effect of viral infection; and *ts*NY72-4 virus for temperature shift experiments (9, 23). Glucose concentration was monitored with glucose oxidase strips (Dextrostix; Miles Laboratories, Inc.).

Construction of cDNA library and recloning. An RSV-transformed CEF cDNA library was constructed by the method of Huynh et al. (14) with 10 μg of oligo(dT)-selected RNA and with λgt10 as the vector. λ Phage DNA was prepared and inserts were recloned into plasmid vectors by standard techniques (21).

RNA preparation, RNA gel electrophoresis, and Northern (RNA) analysis. RNA was prepared by guanidine hydrochloride-ethanol precipitation (2). RNA samples were denatured by heating in formamide-formaldehyde buffer, and 1 μg of ethidium bromide was added to each sample before it was loaded on a formaldehyde agarose gel (3). After electrophoresis, RNA was transferred to a nylon membrane (Zetabind; American Bioanalytical) by capillary transfer. A photograph of the nylon membrane was taken under UV illumination to confirm equal RNA loading and transfer.

Nylon membranes were prehybridized at 42°C for 12 to 24 h in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–10× Denhardt medium–5% dextran sulfate–50 mM sodium phosphate buffer (pH 6.5)–500 μg of denatured salmon sperm DNA per ml. Hybridization was done at 42°C for 24 h in 50% formamide–5× SSC, 1×

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Denhardt medium–10% dextran sulfate–20 mM sodium phosphate buffer (pH 6.5)–100 μg of denatured salmon sperm DNA per ml containing 1×10^6 to 10×10^6 cpm of probe labeled by nick translation per ml. After hybridization, filters were washed with 2× SSC–0.1% sodium dodecyl sulfate (SDS) at 25°C for 30 min, 0.1× SSC–0.1% SDS at 25°C for 30 min, and 0.1× SSC–0.1% SDS at 60°C for 1 h, with two changes of wash solution at each temperature. Filters were exposed to Kodak XAR or BB film with an intensifying screen at −70°C. Autoradiograms were analyzed by densitometric scanning.

Protein analysis. Cytoplasmic P100 fractions were prepared as described elsewhere (16). Protein content was measured with the Bio-Rad Protein Assay. Samples were subjected to SDS-polyacrylamide gel electrophoresis, and proteins were visualized by Coomassie blue staining.

DNA sequencing. M13 phages carrying restriction fragments of R1H cDNA were prepared by standard techniques (21). Nucleotide sequence analysis was performed by the dideoxy chain-termination method (32) with reverse transcriptase or T7 polymerase (Sequenase; U.S. Biochemical Corp.).

RESULTS

Isolation and identification of R1H clone. A CEF cDNA library was constructed in λgt10 with poly(A)$^+$ RNA from RSV-transformed CEF by the methods of Huynh et al. (14). We decided to first identify cDNAs with increased expression in RSV-transformed CEF. Approximately 2,000 recombinant plaques were screened with ^{32}P-labeled cDNA of poly(A)$^+$ RNA from RSV-transformed and uninfected CEF. Plaques that hybridized more strongly to RSV-transformed CEF cDNA were picked for further analysis. These were screened with ^{32}P-labeled viral cDNA, and most were found to contain RSV cDNA. Two recombinant plaques that hybridized more strongly to RSV-transformed CEF cDNA and did not contain viral sequences were obtained. Nucleotide sequence analysis of one insert revealed that it was a cDNA of vimentin, and Northern analysis showed a threefold increase in mRNA levels in RSV-transformed cells (data not shown). Increased expression of vimentin mRNA in RSV-transformed fibroblasts has been described previously (43). In the Northern analysis, the other cDNA, designated R1H, showed markedly elevated expression in RSV-transformed CEF (Fig. 1). The R1H cDNA insert was 2.0 kilobases long and contained a poly(A)$^+$ tract at one terminus and a single long open reading frame that extended to the other terminus. A primer extension study indicated that R1H lacked approximately 400 base pairs at the 5′ terminus (data not shown). To isolate full-length cDNA clones, we rescreened the RSV-transformed CEF cDNA library, first with R1H cDNA as a probe and then with a 5′-terminal restriction fragment of the longest cDNA isolated in the first rescreening. The longest cDNA obtained consisted of 2,390 base pairs and contained a single long open reading frame that started at the first 5′ AUG at position 48 and terminated in an amber codon at position 2000. The nucleotide sequence was determined from both strands for the entire cDNA except for 95 base pairs adjacent to the 3′ poly(A)$^+$ tail. This region was sequenced from multiple independent M13 phage isolates containing overlapping restriction fragments. The 5′ and 3′ noncoding sequences are shown in Fig. 2; the complete cDNA sequence has been sent to the GenBank database. A polyadenylylation signal, AAUAAA, is present at position 2305 and is followed by a poly(A)$^+$ tract at position 2326.

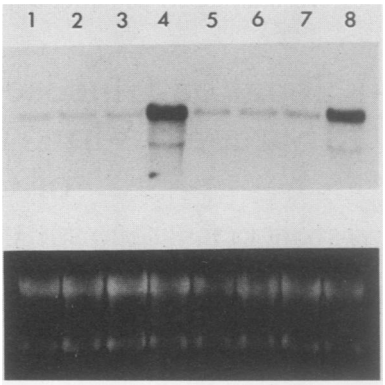

FIG. 1. Northern analysis of GRP78 mRNA in RSV-transformed and uninfected CEF. Cytoplasmic RNA samples were separated by electrophoresis on a 1% agarose formaldehyde gel, transferred to a nylon membrane, and probed with ^{32}P-labeled R1H cDNA. Each lane contains 10 μg of RNA from fully confluent uninfected CEF (lanes 1 and 5), 50% confluent uninfected CEF (lanes 2 and 6), *td*107-infected CEF (lanes 3 and 7), or RSV-infected CEF (lanes 4 and 8). CEF cultures were grown in standard media (lanes 1-4) or high-glucose media (lanes 5-8). A photograph of the membrane taken after transfer to confirm equal loading and transfer of RNA is shown at the bottom.

The length of the longest R1H cDNA agrees with the R1H mRNA size as estimated by Northern analysis and by primer extension study. The single long open reading frame encodes a protein of 652 amino acids with a predicted M_w of 72,020. Nearly one-third (208/652) of the amino acids are charged residues; there are 97 positively charged residues (arginine, histidine, and lysine) and 111 negatively charged residues (aspartic acid and glutamine) (Fig. 3). The overall protein is hydrophilic, according to the parameters of Kyte and Doolittle (17), but has a highly hydrophobic N terminus. This N-terminal hydrophobic region is consistent with known signal peptide sequences (40).

Comparison of the amino acid sequence encoded by R1H mRNA with Dayhoff protein sequence files revealed significant similarity to heat shock proteins from many species. Using this result as a guide, we found that R1H protein was nearly identical to a recently published sequence of rat GRP78 (25), which is also known as immunoglobulin heavy-chain binding protein (8). Of 654 residues in rat GRP78, R1H differs at only 27 positions (96% identity) (Fig. 3). Of the 27 substitutions, 10 lie within the predicted signal peptide, which is also present in rat GRP78, and would not be present in the mature protein. No substitutions were found in 428 consecutive residues that constitute the central two-thirds of the protein. The nucleotide sequences of R1H and rat GRP78 are 84% identical in the coding region, and significant similarity is also present in the 3′ untranslated region (Fig. 2B). A C-terminal amino acid sequence, KDEL, which confers localization in the endoplasmic reticulum (ER) for rat GRP78 (26), is conserved in R1H. The extreme similarity of the amino acid sequences of R1H and rat GRP78 indicates that R1H is the chicken gene for GRP78.

GRP78 expression in RSV-transformed CEF. The level of GRP78 mRNA was markedly elevated in RSV-transformed CEF compared with uninfected cells or cells infected with transformation-defective virus (Fig. 1). We examined whether this increase in mRNA level was simply a consequence of glucose depletion in the media of transformed cell cultures. CEF were grown in standard medium containing 6 mM glucose or in supplemented medium containing 24 mM

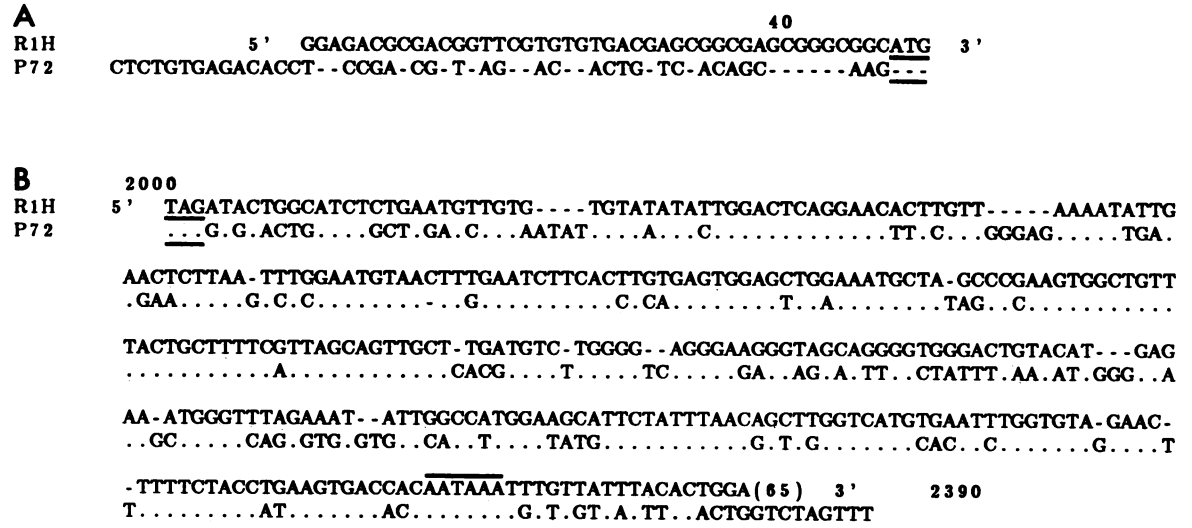

FIG. 2. Nucleotide sequences of the 5′ noncoding region (A) and 3′ noncoding region (B) of R1H compared with analogous sequences of rat GRP78 (P72) (24). Gaps introduced to maximize similarity are indicated by spaces, and identical bases in rat GRP78 are marked by periods. The initiator methionine codons and the termination codons in R1H and rat GRP78 are underlined. A polyadenylylation signal sequence in R1H is overlined. The entire nucleotide sequence of R1H has been sent to the GenBank database.

glucose, and the medium was changed every 24 h. Glucose concentration was measured every 24 h, immediately before the medium was changed. In cultures of uninfected or *td*107-infected CEF, the glucose concentration remained at approximately 5 mM when standard medium was used and was above 14 mM when high-glucose medium was used. In cultures of RSV-transformed CEF, the glucose concentration was undetectable at 24 h when standard medium was used but remained at 14 mM or above when high-glucose medium was used. Thus, glucose was completely depleted within 24 h in cultures of RSV-transformed CEF grown in standard medium. Glucose levels remained elevated, however, in the RSV-transformed cultures grown in high-glucose medium.

We then assayed GRP78 mRNA in these cells. As expected, we found that GRP78 mRNA was induced in RSV-transformed CEF grown in standard medium. The level of GRP78 and mRNA was approximately 10-fold higher in RSV-transformed CEF than in uninfected or *td*107-infected CEF under these conditions (Fig. 1). An unanticipated result was that GRP78 mRNA was induced in RSV-transformed CEF grown in high-glucose medium, in which glucose depletion did not occur. Under these circumstances, GRP78 mRNA was approximately fivefold higher in RSV-transformed compared with uninfected CEF (Fig. 1). No differences in GRP78 mRNA expression were found between stationary and growing cultures of uninfected CEF (Fig. 1).

To determine whether these changes in mRNA levels were

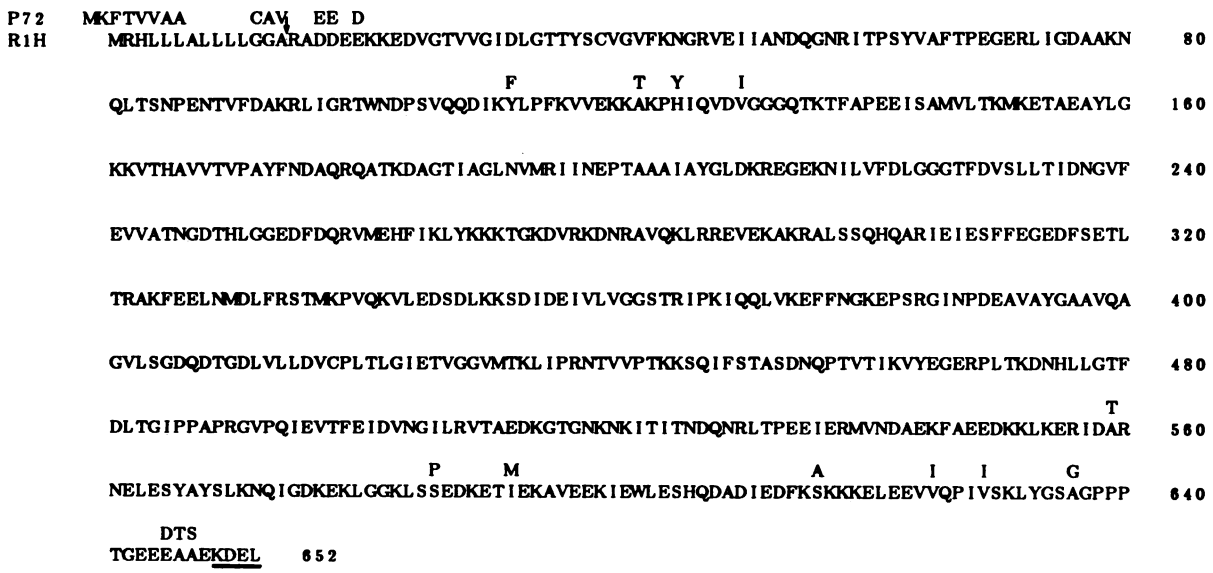

FIG. 3. Amino acid sequence of R1H compared with that of rat GRP78. The complete predicted amino acid sequence of R1H is shown (R1H). For rat GRP78 (P72) (24), only the residues that differ from those of R1H are shown. An arrow indicates the most likely signal peptide cleavage site as predicted by hydropathy plot (16) and comparison with known signal peptides (41). A C-terminal ER localization signal sequence that is found in mammalian proteins (25) is underlined.

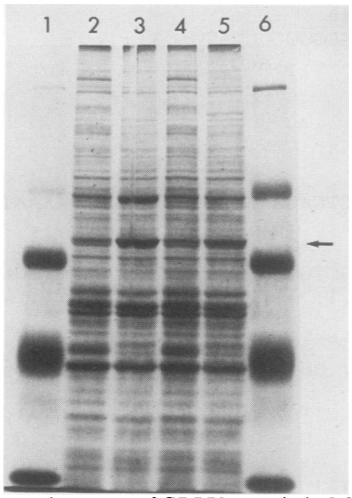

FIG. 4. Increased content of GRP78 protein in RSV-transformed CEF grown in standard and high-glucose media. Cytoplasmic P100 fractions of uninfected and RSV-infected CEF were prepared and separated on a 7.5% polyacrylamide gel, and proteins were visualized by Coomassie blue staining. Each lane contains 80 μg of protein from uninfected CEF (lanes 2 and 4) or RSV-infected CEF (lanes 3 and 5) that were grown in standard medium (lanes 2 and 3) or high-glucose medium (lanes 4 and 5). Lanes 1 and 6 contain protein molecular size markers (Bethesda Research Laboratories, Inc.) of 205, 103, 67, 42, and 28 kilodaltons. An arrow marks the position of GRP78.

reflected in protein content, we first enriched for GRP78 content by preparing the cytoplasmic P100 fraction, which is known to contain essentially all of the cellular GRP78 as well as GRP94 (35). Samples were analyzed by SDS-polyacrylamide gel electrophoresis, and proteins were visualized by Coomassie blue staining. In comparing RSV-transformed and uninfected CEF grown in standard medium, we found two bands that were markedly increased in RSV-transformed cells (Fig. 4), consistent with the induction of GRP78 and GRP94 expression (36). In CEF grown in high-glucose medium, the 78-kilodalton band was less prominent in RSV-transformed CEF but still clearly elevated compared with the band in uninfected CEF. Under these conditions, the 94-kilodalton band was not increased in RSV-transformed CEF compared with uninfected CEF.

To examine the time course of v-*src* induction of GRP78 synthesis, we infected CEF with an RSV mutant expressing temperature-sensitive $p60^{v\text{-}src}$ (*ts*NY72-4) and examined GRP78 mRNA levels before and after shift down to permissive temperature in glucose-supplemented medium. The glucose concentration in the medium was assayed at each time point and remained above 14 mM in RSV-transformed and uninfected CEF. We found that the level of GRP78 mRNA was slightly elevated at 4 h after shift down to permissive temperature and increased gradually over 48 h (Fig. 5). No change was seen in uninfected CEF grown under these conditions (Fig. 5).

One essential function of glucose metabolism in HeLa cells is to provide ribose as a precursor for nucleotides (30). The need for ribose-phosphate can be met by supplementing medium with 5 mM uridine (42). These observations suggested to us that changes in glucose utilization in transformed cells might limit nucleotide synthesis and that increased expression of GRP78 in RSV-transformed cells might be suppressed by uridine supplementation. When CEF infected with a temperature-sensitive mutant were shifted

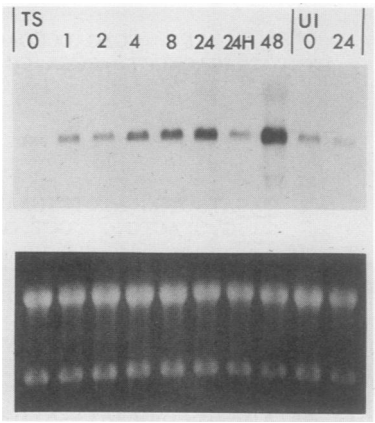

FIG. 5. Increased level of GRP78 mRNA in *ts*NY72-4-infected cells after shift down to permissive temperature. Cytoplasmic RNA from *ts*NY72-4-infected CEF (TS) and uninfected CEF (UI) was collected at indicated times after shift down to permissive temperature and analyzed as described in the legend to Fig. 1. Numbers indicate hours after shift down to permissive temperature. Supplemental glucose was added to the media to maintain glucose concentration greater than 14 mM. Otherwise the media were not changed during the course of the experiment. A photograph of the nylon membrane after transfer is also shown at the bottom.

down to permissive temperature in medium containing 5 mM uridine and either 0 or 25 mM glucose, the level of GRP78 mRNA increased approximately fivefold over 48 h (Fig. 6). Thus, the time course and the final level of GRP78 mRNA did not appear to be altered by uridine supplementation.

DISCUSSION

RSV-transformed CEF differ markedly from normal cells in morphology, metabolic activity, and growth characteristics (for a review, see reference 10). The extent to which particular phenotypic features of RSV-transformed cells reflect alterations in the expression of cellular genes is not known. Given the plethora of phenotypic alterations found

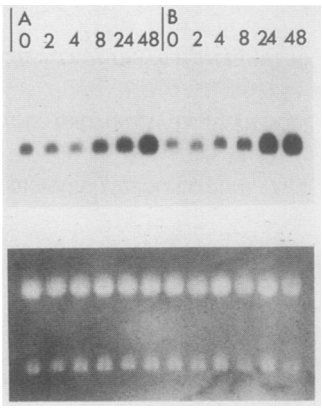

FIG. 6. Effect of uridine supplementation on the induction of GRP78 mRNA in *ts*NY72-4-infected CEF after shift down to permissive temperature. At time of temperature shift down, the medium was changed to RPMI-1640 medium containing 5 mM uridine and either 0 mM glucose (A) or 24 mM glucose (B). Numbers indicate hours after temperature shift. In each lane, 3 μg of RNA was loaded and analyzed by the Northern procedure as described in the text. A photograph of the filter after transfer is shown at the bottom.

in RSV-transformed cells, it is perhaps surprising that few genes whose expression is changed after transformation have been identified. Cellular mRNAs with increased expression in RSV-transformed fibroblasts include β-globulin (7), transin (22), cathepsin (6), and glucose transporter protein (5, 34); mRNAs with decreased expression include fibronectin (4), α-1 and α-2 collagen (12), and tropomyosin (11). Most of these genes encode proteins that were known to be changed in content or activity after transformation and were assayed for mRNA expression when cDNA clones became available. We have taken an alternative systematic approach to identifying cellular genes whose expression is changed after transformation by screening cDNA libraries by differential hybridization with pooled cDNA probes from normal and RSV-transformed CEF. More than 95% of the recombinant plaques screened in this study hybridized equally well to cDNA probes synthesized from poly(A)$^+$ RNA from uninfected and RSV-transformed CEF. This suggests that the expression of most of the more abundant mRNAs is unchanged after transformation. In this paper we report the isolation of a cDNA clone R1H for which the mRNA level is 20-fold higher in RSV-transformed cells. Nucleotide sequence analysis revealed that R1H encodes a heat shock family protein, GRP78. We also report the regulated expression of GRP78 mRNA in RSV-transformed and uninfected CEF.

Our results that show increased expression of GRP78 in RSV-transformed CEF grown in standard medium are consistent with earlier reports. GRP78 was first identified as one of two proteins induced in RSV-transformed CEF (15, 37). Subsequently it was shown that glucose deprivation could induce GRP78 synthesis in normal cells and that the increase in GRP78 protein after transformation could be suppressed by glucose supplementation, and it was concluded that the increase in GRP78 protein in RSV-transformed cells was a consequence of rapid glucose depletion (36). In study presented here, we found that the level of GRP78 mRNA and the GRP78 protein content were increased in RSV-transformed CEF, even in the absence of glucose deprivation. Our findings indicate that induction of GRP78 synthesis in RSV-transformed CEF is in part a response to an alteration in the transformed cell other than glucose deprivation.

It is striking that the primary amino acid sequence of chicken GRP78 is nearly identical to that of rat GRP78. Excluding the putative signal peptides, the two proteins differ at only 17 of 638 positions (97% identity), and 8 of these are conservative substitutions. GRP78 is more highly conserved than HSP70, which is a related heat shock family protein. Chicken HSP70 and human HSP70 differ at 109 of 634 positions (83% identity) (13, 24). We found that 46% of the codons that encode residues which are conserved between chicken and rat GRP78 genes contain silent mutations. This proportion of silent mutations is similar to that in other conserved genes (Table 1), which suggests that the extreme conservation of GRP78 reflects selective pressure at the protein level. GRP78 may interact with cellular components that are also highly conserved.

GRP78 is a heat shock family protein that is constitutively expressed in normal cells, is resident in the ER (25, 35), and is thought to function in retaining abnormal proteins in the ER, such as incompletely glycosylated proteins (for reviews, see references 18 and 28). Increased expression of GRP78 after transformation may then reflect increased traffic of such proteins in the ER. Increased expression of GRP78 is found in paramyxovirus-infected cells (29), and this is thought to be a response to either viral glycoproteins or disruption of normal cellular glycosylation by infection. Retroviral infection cannot explain the induction seen in RSV-transformed CEF, because GRP78 mRNA levels were not elevated in cells infected with transformation-defective virus (Fig. 1) and were induced in *ts*NY72-4-infected cells only at the permissive temperature (Fig. 5 and 6).

TABLE 1. Silent mutations in chicken and rat genes

Gene	Codon ratio[a] (%)	References
GRP78	285/619 (46)	This paper; 25
Cytochrome *c*	32/65 (49)	20, 33
α-Tubulin	167/409 (41)	19, 39

[a] Number of codons with silent mutations/number of codons encoding residues conserved between chicken and rat genes.

In CEF infected with *ts*NY72-4 virus, elevated levels of GRP78 mRNA were found beginning at 4 h after shift down to a permissive temperature (Fig. 5). The time course of induction suggests that increased expression of GRP78 is not a primary effect of p60$^{v\text{-}src}$ activity but is a secondary response to other metabolic changes that occur in the transformed cell. In support of this, we found that when cells infected with a temperature-sensitive mutant were treated with cycloheximide, GRP78 and mRNA levels were much lower and there was no increase following temperature shift down (data not shown). Because the expression of GRP78 is thought to be linked to changes in protein traffic in the ER, it is not surprising that inhibition of protein synthesis was followed by a decline in GRP78 expression. These findings on the induction of GRP78 by p60$^{v\text{-}src}$ are similar to those for other known stimuli of GRP78, including calcium ionophore and glycosylation inhibitors (27, 31, 41). These stimuli require several hours for maximal effect and are blocked by cycloheximide, which suggests that the proximate stimulus for transcription has not yet been identified.

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