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Highly conserved glucose-regulated protein in hamster and chicken cells: Preliminary characterization of its cDNA clone

(peptide mapping/blot hybridization/hybrid-select translation)

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ABSTRACT **A temperature-sensitive mutant K12 derived from a Chinese hamster fibroblast has been shown to overproduce three specific proteins of M_r 94,000, 78,000, and 58,000 when incubated at the nonpermissive temperature (40.5°C). We previously identified these proteins as glucose-regulated proteins similar to those observed in chicken embryo fibroblasts when the cells are starved of glucose. In this report, we show that the M_r 78,000 proteins isolated from the hamster K12 cell line and from chicken embryo fibroblasts have identical electrophoretic mobilities in two-dimensional isoelectric focusing gels and nearly identical peptide maps. However, these proteins are different from heat-shock proteins previously described for animal cells. We have constructed a library of cDNA clones by using the RNA extracted from the hamster K12 cells incubated at 40.5°C. Clones that hybridize preferentially with cDNA made from RNA at 40.5°C were selected. By using the hybrid-selection technique, followed by *in vitro* translation, a cDNA clone containing a 2550-nucleotide insert coding for the hamster M_r 78,000 protein has been identified.**

It has been observed in various animal cell lines that, when the glucose in the growth medium is depleted, the synthesis of a specific set of proteins is enhanced (1–4). Two of these proteins observed in chicken embryo fibroblasts have M_rs of 90,000–95,000 and 73,000–79,000 and were originally thought to be transformation-specific proteins (5, 6). Subsequently, it was demonstrated that increased contents of these proteins were actually due to rapid depletion of glucose from the growth medium by the transformed cells (1). The accumulation of three specific proteins (M_r 94,000, 78,000, and 58,000) related to the glucose-regulated proteins in a temperature-sensitive hamster mutant cell line, K12, has been reported (4). This cell line is particularly useful for the study of the coordinated expression of these proteins because, when the cells are shifted to the nonpermissive temperature (40.5°C), the increase in synthesis of the glucose-regulated proteins is 5-fold to 10-fold greater than that obtained by glucose starvation. This accumulation is primarily due to new transcription and synthesis (7). Thus, the hamster K12 cells provide a convenient system for the molecular cloning of the genes coding for these proteins.

In this report we describe experiments which characterize the relationship of the M_r 78,000 glucose-regulated proteins produced by hamster and chicken embryo fibroblasts. Our results indicate that this protein is highly conserved in the two species. Contrary to an earlier speculation (8), we demonstrate that the glucose-regulated proteins are different from heat-shock proteins observed in animal cells. We also present preliminary evidence for the identification of a cDNA clone coding for the M_r 78,000 glucose-regulated protein.

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MATERIALS AND METHODS

Cell Lines and Culture Conditions. Conditions for culturing the hamster cell lines and labeling the cellular proteins have been described (4). For the preparation of total cell lysate, the cell pellet was immediately resuspended in lysis buffer [8 M urea/2% (wt/vol) Nonidet P-40/0.5% 2-mercaptoethanol]. Primary cultures of chicken embryo fibroblasts were a gift from Michael Lai and were maintained in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% calf serum.

Isolation of M_r 78,000 Proteins for Peptide Mapping. [^{35}S]-Methionine-labeled total cell lysates were prepared from hamster mutant K12 cells incubated at 40.5°C for 16 hr or from third-passage chicken embryo fibroblasts grown in glucose-free medium (4) at 35°C for 24 hr. The samples were mixed with ≈100 μg of unlabeled cell lysate and resolved by two-dimensional gel electrophoresis. The gels were quick-stained for 30 min in 0.1% Coomassie blue in 50% methanol/10% acetic acid, and the spot containing the M_r 78,000 protein was excised from each gel. The protein samples were recovered from the gel by electroelution into a dialysis bag. After dialysis against 0.05 M ammonium bicarbonate/6 M urea to remove $NaDodSO_4$, followed by dialysis against 0.05 M ammonium bicarbonate, the samples were lyophilized. Prior to digestion with *Staphylococcus aureus* protease V8 (Miles), the samples were resuspended in peptide buffer [0.125 M Tris, pH 6.8/0.5% $NaDodSO_4$/10% (vol/vol) glycerol].

Construction of cDNA Clones. K12 cells were grown to confluence at 35°C and incubated at 40.5°C for 16 hr. Cytoplasmic RNA was prepared from these cells by lysis in 0.5% Nonidet P-40 as described (9). The poly(A)$^+$RNA fraction was prepared by chromatography on oligo(dT)-cellulose. These fractions were used as templates for cDNA synthesis. The poly(A)$^+$RNA·cDNA hybrid was tailed with oligo(dA) by using terminal transferase. Plasmid pBR322 was cleaved with *Bam*HI and similarly tailed with oligo(dT). The RNA·cDNA hybrid was cloned into pBR322 by the method of Zain *et al.* (10). Transformation was by the procedure of Kushner (11) into HB101. Ninety percent of the transformants contained recombinant plasmids. About 500 cDNA clones were screened for sequences differentially expressed by K12 cells at 40.5°C. Colony hybridization procedures were performed essentially as described by Lasky *et al.* (12), except both the prehybridization and hybridization mixtures contained 4× SET instead of 1× SET (1× SET = 0.15 M NaCl/0.03 M Tris, pH 8/2 mM EDTA as described in ref. 12), and 50 μg of poly(rC) was also included.

Translation of Hybrid-Selected RNA. The conditions for hybrid-selected translation were described by Ricciardi *et al.* (13). Two micrograms of the recombinant plasmid DNA was baked onto nitrocellulose filters and incubated with 2 μg of poly(A)$^+$RNA extracted from K12 cells incubated at 40.5°C.

Hybridization was carried out at 50°C for 4 hr. The bound RNA was eluted by boiling the filters for 90 sec in 300 μl of 1 mM EDTA (pH 6) containing rabbit liver tRNA (15 μg/ml) as carrier. The RNA was translated in the rabbit reticulocyte system (Bethesda Research Laboratories, Rockville, MD) supplemented with [^{35}S]methionine (Amersham; 980 Ci/mmol; 1 Ci = 3.6×10^{10} becquerels). *In vitro* translation products were analyzed on polyacrylamide gels.

RESULTS

Glucose-Regulated Proteins Are Different from Heat-Shock Proteins. It has been reported (8) that a mild heat-shock at 45°C causes certain animal cells to increase synthesis of a set of proteins. Those authors found the M_rs of those proteins to be similar to M_rs of glucose-regulated proteins (2) and suggested that the two sets of proteins are similar. In order to determine if these proteins are identical, we pulse-labeled proteins synthesized by the hamster mutant cell line K12 as well as by its nonmutant parent cell line, Wg1A (14), at 40.5°C. The proteins were analyzed on two-dimensional isoelectric focusing polyacrylamide gels (Fig. 1 *A* and B). We found that in hamster cells the glucose-regulated proteins are different from the heat-shock proteins in the following ways. (*i*) The M_rs of the glucose-regulated proteins are 94,000, 78,000, and 58,000, whereas the major heat-shock protein species has a M_r of about 73,000. (*ii*) The pI values of the hamster M_r 73,000 heat-shock proteins are around 5.6 and are slightly more basic than the pI of actin, similar to that observed for the M_r 68,000–70,000 major heat-shock proteins in *Drosophila* (15). The pI values exhibited by the M_r 94,000, 78,000, and 58,000 glucose-regulated proteins are 5.1, 5.3, and 5.9, respectively (4). (*iii*) The induced synthesis of the heat-shock proteins is immediate but lasts only a few hours. In contrast, the synthesis of the glucose-regulated proteins continues for at least 24 hr.

Comparison of Hamster and Chicken Proteins. Proteins of M_r 90,000–95,000 and 73,000–79,000 have been observed in normal chicken embryo fibroblasts upon removal of glucose from the growth medium (1). To determine whether these proteins are identical to the glucose-regulated proteins inducible in hamster cells, proteins synthesized by the hamster K12 and chicken embryo fibroblasts were analyzed on two-dimensional polyacrylamide gels. The M_r 78,000 protein overproduced in K12 cells incubated at 40.5°C was also observed as a major protein species when the same cells were starved of glucose at 35°C (Fig. 1 *A* and *C*). A protein of identical M_r and pI was synthesized in chicken cells when subjected to glucose starvation (Fig. 1*D*).

To investigate if the amino acid sequence of the M_r 78,000 protein found in the hamster and chicken cells was conserved, this protein was purified from two-dimensional gels and subjected to digestion with increasing amounts of protease from *S. aureus*. The hamster and chicken proteins yielded similar peptide fragment maps (Fig. 2). Therefore, at least in chicken and hamster cells, the amino acid sequence of the M_r 78,000 glucose-regulated protein is highly conserved. We also observed that the radiolabeled M_r 78,000 protein is labile. Thus, in some cases a small fraction of the protein will degrade spontaneously into large peptide fragments.

Construction and Screening of cDNA Clones. For the purpose of obtaining cloned probes for these glucose-regulated proteins, a cDNA library was constructed by using poly(A)$^+$RNA extracted from K12 cells incubated at 40.5°C and screened for gene sequences that are differentially expressed at 40.5°C. About 500 cDNA clones were grown in microtiter plates and replicas of the library microtiter plates were imprinted, grown, and amplified on Millipore nitrocellulose filters. Identical sets of filters were hybridized with [^{32}P]cDNA probes; poly(A)$^+$RNA obtained from K12 cells grown at 40.5°C and at 35°C was used as templates. An example of the colony hybridization results is shown in Fig. 3. Clones that hybridized intensely with the 40.5°C cDNA probe, but only slightly with the 35°C probe, were selected. The rationale for this screening procedure is that, whereas the M_r 94,000, 78,000, and 58,000 proteins were observed in small amounts at 35°C, they were the major proteins being synthesized at 40.5°C (4).

Fig. 4 shows a restriction map of one such selected cDNA clone, 3C5, which was subsequently found to contain the sequence coding for the M_r 78,000 protein (see below). The size

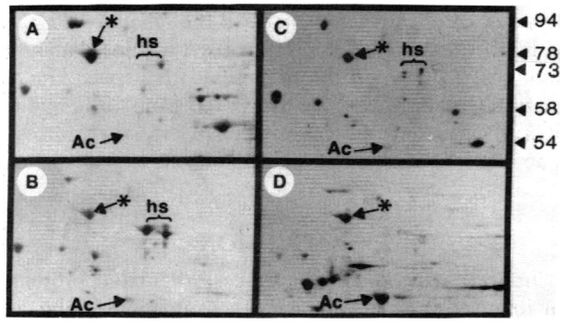

FIG. 1. Two-dimensional gel electrophoresis and autoradiography of hamster and chicken embryo fibroblast proteins. (*A*) K12 cells labeled for 8 hr at 40.5°C. Prior to the labeling period, the cells had been incubated at 40.5°C for 16 hr. (*B*) Wg1A cells labeled for 8 hr at 40.5°C. The cells were labeled immediately after shift from 35°C. (*C*) K12 cells labeled for 24 hr in glucose-free medium at 35°C. (*D*) Third-passage chicken embryo fibroblast cells labeled for 24 hr in glucose-free medium at 35°C. M_rs ($\times 10^{-3}$) are shown at the right. After each labeling period, total cell lysate was prepared. Approximately 5×10^5 cpm of the [^{3}H]leucine-labeled protein was applied to each gel. The concentration of polyacrylamide in the running gel was 8.5%. Isoelectric focusing was from right (basic) to left (acidic) in the gels presented; $NaDodSO_4$ gel was from top to bottom. *, M_r 78,000 glucose-regulated protein; hs, heat-shock proteins; Ac, actin. Conditions for gel electrophoresis and fluorography were as described (4).

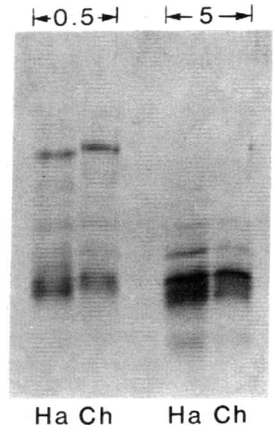

FIG. 2. Partial proteolytic digests of the M_r 78,000 proteins from hamster (Ha) and chicken (Ch) cells. [^{35}S]Methionine-labeled M_r 78,000 protein from K12 cells incubated at 40.5°C or from chicken embryo fibroblasts grown in glucose-free medium at 35°C was purified by two-dimensional gel electrophoresis. After electroelution, the proteins were digested with *S. aureus* protease V8 (0.5 and 5 μg per lane) by the procedure of Cleveland *et al.* (16). The resultant peptides were analyzed on a 13.5% polyacrylamide gel. The autoradiogram of the gel after fluorography is shown.

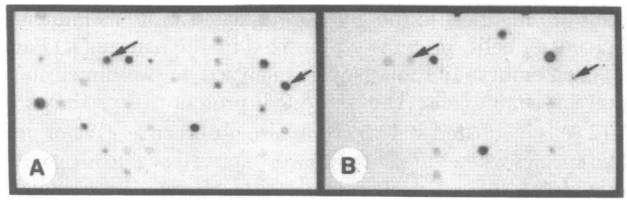

FIG. 3. Detection of cDNA clones by colony hybridization. The autoradiograms from a set of replicated plates after hybridization with [^{32}P]cDNA synthesized by using, as template, poly(A)$^+$RNA isolated from K12 cells incubated at 40.5°C (*A*) or 35°C (*B*). Arrows, colonies demonstrating preferred hybridization to the 40.5°C probe but also detectable at low levels when hybridized with the 35°C probe.

of the cDNA insert of 3C5, as determined by gel electrophoresis, is estimated to be ≈2550 nucleotides. The restriction patterns of 3C5 and three other selected cDNA clones after digestion with *Hin*dIII and *Sal* I are shown in Fig. 5*A*. When these restriction fragments were blotted onto nitrocellulose paper by the method of Southern (18) and hybridized with [^{32}P]cDNA from template poly(A)$^+$RNA extracted from K12 cells incubated at 40.5°C, the 3C5 insert hybridized more intensely than other clones (Fig. 5*B*). Thus, 3C5 contains a sequence complementary to a highly abundant RNA species in K12 cells when incubated at 40.5°C and therefore is a likely candidate for containing the gene sequences coding for one of the three overproduced K12 proteins.

Identification of Protein Coded for by cDNA Clone. In order to determine the protein coded for by cDNA clone 3C5, its complementary mRNA sequence was isolated by filter hybridization. Poly(A)$^+$RNA prepared from K12 cells incubated at 40.5°C was hybridized to filter-bound plasmid DNA. The hybridized mRNA was isolated by boiling and then translated in a rabbit reticulocyte lysate cell-free translation system in the presence of [^{35}S]methionine. The results (Fig. 6) demonstrated that the cloned 3C5 sequence selectively hybridized with a mRNA species that codes for a protein similar in M_r, as well as pI, to the M_r 78,000 glucose-regulated protein. This hybrid-selected

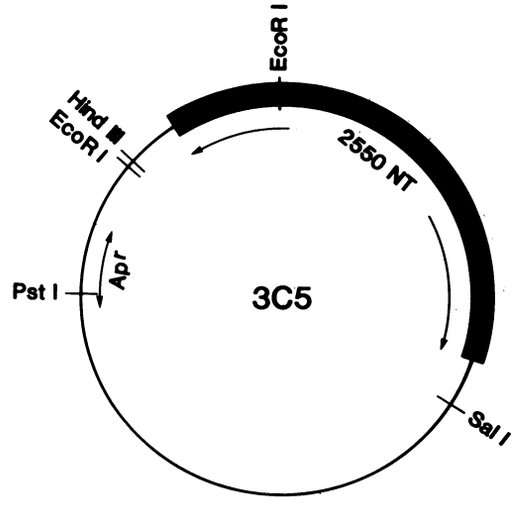

FIG. 4. Restriction map of cDNA clone 3C5. The cDNA insert (heavy line) was introduced into the unique *Bam*HI site of pBR322 (17). The *Bam*HI site and the tetracycline-resistance gene were destroyed in the preparation of the vector when approximately 100 residues of dT were tailed at its 3′ end with terminal transferase. To isolate the cDNA inserts, the plasmids can be digested with *Hin*dIII and *Sal* I. The insert carries approximately 510 nucleotides of the pBR322 sequence. The cDNA insert, including the poly(A·T) tails, is estimated to be 2550 nucleotides long.

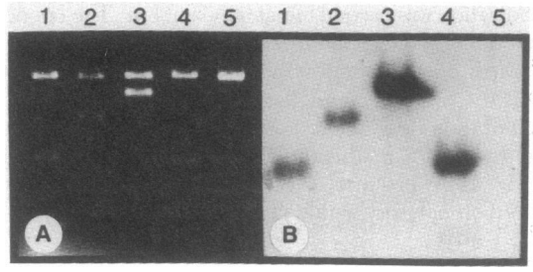

FIG. 5. Hybridization of cloned DNA insert with [^{32}P]cDNA. (*A*) About 1 μg of each plasmid DNA was digested with *Hin*dIII and *Sal* I and separated by electrophoresis on 1% agarose gel. The gel was stained with ethidium bromide 1 μg/ml. Lanes: 1, clone 1B; 2, clone 2F10; 3, clone 3C5; 4, clone 4A3; 5, pBR322. (*B*) The gel in *A* was alkali-treated and the DNA fragments were transferred to nitrocellulose paper as described (18). Conditions for the hybridization with [^{32}P]cDNA prepared by using poly(A)$^+$RNA extracted from K12 cells incubated at 40.5°C were as follows: 10^6 cpm of [^{32}P]cDNA (≈5 × 10^6 cpm/μg) was added to 10 ml of hybridization buffer and incubated at 68°C for 16 hr; the nitrocellulose paper was extensively washed and autoradiographed overnight.

translation product was absent from the control sample showing endogenous synthesis products in the reticulocyte translation system. Identical results were obtained when the 3C5 insert DNA alone was used for the RNA hybrid-selection.

When total K12 poly(A)$^+$RNA was translated in cell-free systems (including both wheat germ and reticulocyte), the *in vivo* M_r 78,000 protein was always translated as a protein species of slightly higher molecular weight and slightly more basic pI. These minor differences probably are due to posttranslational modifications of this protein, which are deficient in cell-free or heterologous systems (19, 20). In the two-dimensional gels, sometimes a streak or a minor spot could be observed directly underneath the M_r 78,000 protein (Fig. 6*B*). This probably represents degradation products of the M_r 78,000 protein, due to its labile nature, and was observed only when high specific activity [^{35}S]methionine was used to label the protein.

Based on the results of hybrid-selection translation, it can be concluded that the cDNA clone 3C5 contains the sequence coding for the M_r 78,000 protein. Consistent with this finding are three observations we have made: (*i*) the labeled 3C5 plasmid DNA hybridizes with a cytoplasmic RNA species of about 2750 nucleotides; (*ii*) the concentration of this RNA species increases dramatically when K12 cells are shifted from 35°C to 40.5°C; and (*iii*) the labeled 3C5 plasmid DNA cross-hybridizes with unique restriction fragments of chicken genomic DNA (unpublished data).

DISCUSSION

The induced synthesis of the glucose-regulated proteins has been reported for chicken, hamster, rat, mouse, and human cells. We believe that this response of animal cells to glucose starvation is a common phenomenon and is important for cell survival under this condition. Of particular interest is the function served by these glucose-regulated proteins. It has been suggested that they may be involved in hexose transport (2) or perform a specialized function related to protein glycosylation (21). Suggestions have also been made that the glucose-regulated proteins may possibly be a component of the insulin receptor (3, 22). Interestingly, the M_r 58,000 protein is of the same M_r as the glucose transporter protein (23), and a set of proteins similar in M_r to the K12 glucose-regulated proteins (90,000, 75,000, and 56,000) have been found to copurify with the insulin receptor (24, 25). The glucose-regulated proteins characterized so far in other systems are reported to be located in plasma

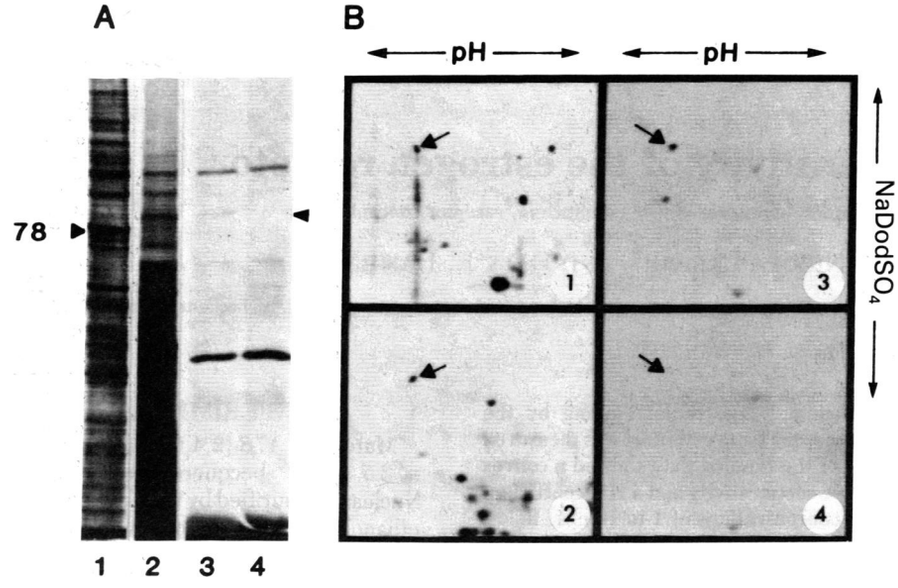

FIG. 6. Analysis of *in vivo*-labeled and *in vitro*-translated proteins by slab gel electrophoresis (*A*) and two-dimensional gel electrophoresis (*B*). (*A*) Lanes: 1, *in vivo* [^{35}S]methionine-labeled K12 proteins; 2, *in vitro* translation products using as template total poly(A)$^+$RNA extracted from K12 cells incubated at 40.5°C; 3, translation products of mRNA selected by hybridization to cloned 3C5 sequence; 4, translation products obtained when no exogenous RNA was added to the reticulocyte system. Autoradiograms of the gels are shown. Position of the M_r 78,000 protein is shown. (*B*) Isoelectric focusing was in the horizontal direction (pH 5 at left; pH 7 at right); $NaDodSO_4$/polyacrylamide gel electrophoresis was in the vertical direction.

membranes. However, we have found that the cellular localization of the M_r 78,000 protein overproduced in hamster K12 cells is ubiquitous. In any case, the remarkable conservation of the primary sequence of this protein among the two widely divergent animal species argues that its function is of major physiological significance. Furthermore, this protein is found in detectable amounts when the cells are growing under normal culture conditions (4), implying that it is required for normal cell growth.

Our results indicate that, in hamster cells, the major heat-shock proteins are different from the glucose-regulated proteins both in M_r and in pI. On the other hand, the M_r 73,000 hamster heat-shock proteins we observed bear striking resemblance, both in M_r and pI, to the M_r 71,000–72,000 proteins accumulated in cultured chicken cells exposed to the arginine analogue canavanine (26).

cDNA clones containing the sequence coding for the glucose-regulated proteins provide a unique tool for studying the differential and coordinated expression of this set of proteins in animal cells. By using cloned 3C5 DNA as a probe, it is now possible to determine the genomic organization of the M_r 78,000 protein in both hamster and chicken cells.

Note Added in Proof. The *in vivo* M_r 78,000 protein and the *in vitro* translated protein yield the same peptide maps.

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