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Rapid Repression of Quiescence-Specific Gene Expression by Epidermal Growth Factor, Insulin, and pp60$^{v\text{-}src}$

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We isolated a cDNA for p20K, a secreted protein preferentially synthesized in nonproliferating cells. p20K mRNA and protein levels declined rapidly following treatment with various mitogens. DNA sequence analysis of the p20K cDNA predicted a novel protein distantly related to $\alpha_{2\mu}$-globulin and plasma retinol-binding protein.

Numerous studies focused on gene expression have led to the identification of genes induced within minutes after the mitogenic stimulation of cells (12, 16, 17). In sharp contrast to this mode of gene expression, we have recently identified gene products in quiescent chicken embryo fibroblasts (CEF) and chicken heart mesenchymal (CHM) cells that are not expressed in mitogen-stimulated or Rous sarcoma virus (RSV)-transformed cells (7). We have now obtained a cDNA clone encoding a major quiescence-specific gene product in these cells, p20K, and demonstrated that, in contrast to the situation with a large set of genes, the levels of p20K mRNA decline rapidly within a few hours after mitogen stimulation or transformation by RSV. Basal (repressed) levels of p20K mRNA are attained during the G_1 phase of the cell cycle, well before the onset of cell division. Hence, repression of p20K synthesis coincides with the expression of the "immediate-early" mitogen response gene CEF-4. The amino acid sequence of p20K deduced from cDNA clones indicates a limited similarity to $\alpha_{2\mu}$-globulin, β-lactoglobulin, and plasma retinol-binding protein.

To isolate cDNA clones encoding p20K, we constructed a cDNA library representing sequences expressed in contact-inhibited CEF in lambda ZAP (Stratagene, San Diego, Calif.). We previously reported that quiescent CHM cells cultured in plasma and density-arrested CEF express a specific set of polypeptides (7). An antibody raised against the major quiescence polypeptide, p20K (7), was used to screen the expression library. Seven positive recombinants were isolated from an unamplified pool of 150,000 recombinants. Phagemids were generated by coinfection with R408 helper phage. Several of the seven isolates were found to produce an isopropyl-β-D-thiogalactopyranoside-inducible protein of 25 kilodaltons (kDa) (data not shown). This fusion protein was specifically recognized by the p20K antiserum, suggesting that full-length cDNA clones encoding p20K had been isolated. Positive identification of one of the clones, designated p20K-20, was established by two independent methods (Fig. 1).

In the first approach, polyadenylated RNA isolated by positive hybrid selection with p20K-20 was translated in a rabbit reticulocyte cellular translation system and immunoprecipitated with anti-p20K serum (Fig. 1A). Polyadenylated RNA (25 μg) purified from quiescent CHM cells (7) was hybridized to linearized plasmid DNA bound to nitrocellulose paper (BA85; Schleicher & Schuell, Inc., Keene, N.H.) in 50% formamide–0.6 M NaCl–0.01 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) (pH 6.4)–0.1% sodium dodecyl sulfate for 4 h at 48°C (8). After repeated washes in 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate–2 mM EDTA at 60°C, bound RNA was eluted by boiling for 60 s in a solution of 2 mM EDTA and 30 μg of yeast tRNA per ml. The RNA was recovered by ethanol precipitation and translated in a rabbit reticulocyte cellular translation system containing [^{35}S]methionine (New England Nuclear Corp., Boston, Mass.). The translation products were immunoprecipitated with p20K antiserum 601 (7) (Fig. 1A, lanes 1 to 3) or the corresponding preimmune serum (Fig. 1A, lane 4) and analyzed by polyacrylamide gel electrophoresis and fluorography. A translation product of 22 kDa was detected with this approach (Fig. 1A, lane 1). This polypeptide was not synthesized when RNA selected with the vector without an insert was translated (Fig. 1A, lane 3) or when plasmids bearing unrelated cDNA inserts were used (data not shown). Furthermore, the 22-kDa polypeptide was recognized in a specific manner by the anti-p20K serum, since no detectable translation product was precipitated by the corresponding preimmune serum (Fig. 1A, lane 4). The same 22-kDa polypeptide was detected when the translation products of total unselected polyadenylated RNA were immunoprecipitated (Fig. 1A, lane 2). Thus, it appears that p20K is initially translated as a 22-kDa protein. This is not surprising, since p20K is a secreted protein and therefore is likely to be synthesized with a signal peptide.

In the second approach, we raised an antiserum against the p20K-20 fusion protein produced in bacteria (antiserum 42) and compared the proteins immunoprecipitated by each antiserum (Fig. 1B). The antiserum was added to the culture medium of quiescent CHM cells metabolically labeled with [^{35}S]methionine (New England Nuclear). Both antisera recognized a protein of 20 kDa (data not shown). The 20-kDa polypeptide precipitated by each antiserum was subjected to partial degradation with *Staphylococcus aureaus* V8 protease (Fig. 1B). Identical patterns of peptides were observed, indicating that the antisera recognized the same protein. From the results of hybrid selection and immunoprecipitation, we concluded that the p20K-20 clone encodes the quiescence-specific protein previously described (7).

p20K was initially detected in CHM cells cultured in

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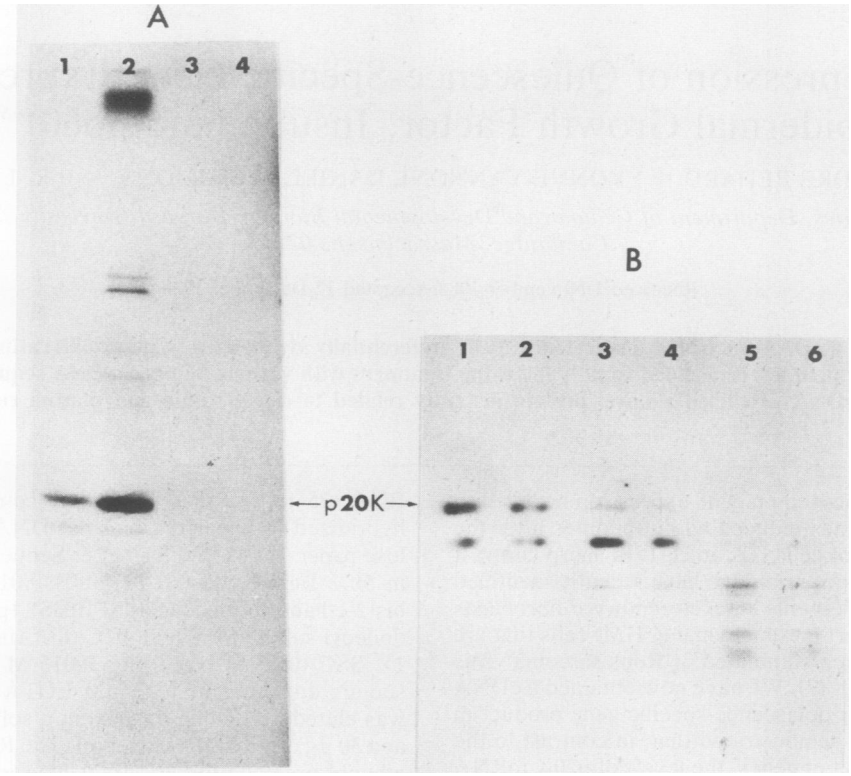

FIG. 1. Characterization of the p20K-20 cDNA clone. (A) Identification by positive hybrid selection and immunoprecipitation. Lane 1, Translation product of RNA selected with the p20K-20 plasmid DNA; lane 2, translation product of total unselected RNA; lane 3, translation product of RNA selected with the vector without an insert. Translation products in lanes 1 to 3 were all immunoprecipitated with an antiserum (denoted 601) raised against the quiescence-specific polypeptide p20K (7). Lane 4, Translation product of RNA selected with the p20K-20 plasmid DNA and immunoprecipitated with the corresponding preimmune serum. (B) Peptide analysis of the 20-kDa protein precipitated by antisera 601 and 42. A fusion protein expressed by the recombinant p20K-20 bacteria was isolated by polyacrylamide gel electrophoresis and injected into the lymph nodes of a New Zealand White female rabbit (22). The resulting antiserum (denoted 42) was compared with the original antiserum (601) raised against isolated p20K protein (7). Radioactively labeled p20K protein secreted by quiescent CHM cells was immunoprecipitated by antisera 601 and 42 and analyzed by limited digestion with *S. aureus* V8 protease (Miles Laboratories Inc., Elkhart, Ind.) (11). p20K immunoprecipitated by antiserum 601 was analyzed in lanes 1, 3, and 5; p20K immunoprecipitated by antiserum 42 was analyzed in lanes 2, 4, and 6. V8 protease was added at 5 ng in lanes 1 and 2, at 50 ng in lanes 3 and 4, and at 500 ng in lanes 5 and 6.

plasma-containing medium (7). In this cell system, quiescence is established without the need for serum starvation (2). In contrast, CEF do not become quiescent in plasma-containing medium under the same conditions but do express p20K when they become confluent and density arrested for cell growth (7).

Rapid proliferation of CHM can be stimulated by the addition of a variety of growth factors, such as epidermal growth factor (EGF) and/or insulin (4), or by transformation by RSV (5). In the latter instance, however, functional $pp60^{v\text{-}src}$ is required, since CHM cells infected with a temperature-sensitive mutant, tsNY72-4RSV, do not proliferate in plasma at the nonpermissive temperature of 41°C (3). Whether stimulation is achieved with growth factors or transformation by RSV, the synthesis of a set of polypeptides specific for the quiescence state (G_0) is repressed in CHM cells shortly after mitogenic stimulation (7).

To investigate the regulation of quiescence gene expression, we characterized the expression of p20K mRNA by Northern (RNA) analysis (Fig. 2). CHM cells were infected with tsNY72-4RSV (provided by H. Hanafusa) and stimulated to proliferate by the addition of EGF or insulin or by the activation of $pp60^{v\text{-}src}$. Early passage CHM cells were infected with the temperature-sensitive mutant tsNY72-4RSV and grown at the nonpermissive temperature of 41°C in Dulbecco modified Eagle medium supplemented with 10% calf serum (HY-CLONE, Logan, Utah). At 90% confluence, the serum-containing medium was replaced by Dulbecco modified Eagle medium containing 5% heat-inactivated, defibrinogenated rooster plasma to establish quiescence (2, 7). After 1 day in plasma, the quiescent CHM cells were either transferred to the permissive temperature of 35°C to activate $pp60^{v\text{-}src}$ (Fig. 2B) or stimulated by the addition of 100 ng of EGF (Biomedical Technologies, Inc., Cambridge, Mass.) per ml or 10,000 ng of insulin (Collaborative Research, Inc., Waltham, Mass.) per ml (Fig. 2A). Under these conditions, the CHM cells divided roughly 16 h after mitogenic stimulation or transformation (data not shown). tsNY72-4RSV-infected CHM cells were grown at the permissive temperature of 35°C in plasma-containing medium (Fig. 2C). To inactivate $pp60^{v\text{-}src}$ and establish quiescence, we transferred the infected CHM cells to the nonpermissive temperature of 41°C. Total RNA was prepared by urea-LiCl precipitation (1) at various time points and analyzed on Northern blots (6). Each filter was probed sequentially with p20K-20, pCEF-4, and GAPDH. All probes were labeled by random oligonucleotide priming (15) and hybridized as described earlier (6). The pCEF-4 clone is induced by expression of the *src* gene product (6). The GAPDH cDNA clone encodes the glyceraldehyde-3-phosphate dehydrogenase gene (7) and was used

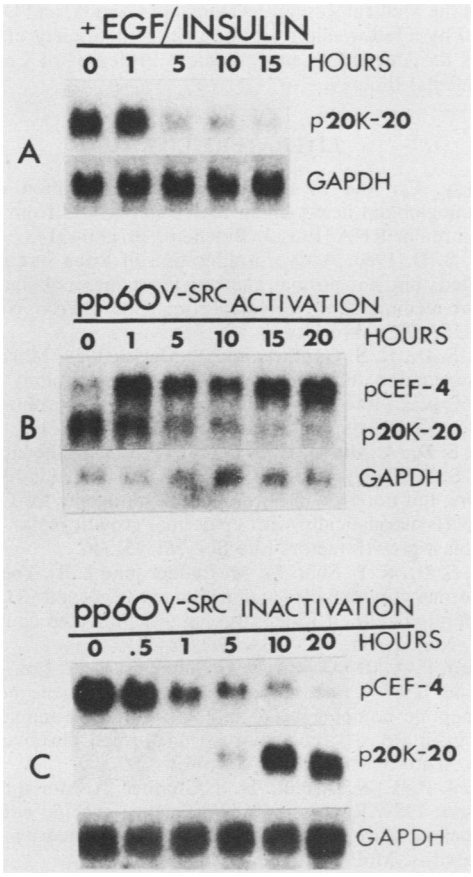

FIG. 2. Regulation of p20K mRNA by pp60$^{v\text{-}src}$, EGF, and insulin in tsNY72-4RSV-infected CHM cells as determined by RNA blot analysis. (A) p20K mRNA in cells after stimulation with EGF and insulin. (B) p20K and pCEF-4 mRNAs in cells after a shift from 41.0 to 35.0°C. (C) p20K and pCEF-4 mRNAs in cells after a shift from 35 to 41.0°C.

to monitor RNA loading. Densitometric scanning was performed with an ULTRASCAN 2202 laser densitometer (LKB Instruments, Inc., Rockville, Md.). Total RNA was prepared from cells stimulated by EGF or insulin at 41°C (Fig. 2A). The level of p20K mRNA decreased rapidly; within 5 h after the addition of EGF or insulin, only basal (repressed) levels of the transcript could be detected. In this instance, complete repression occurred only in the G_1 transition, simultaneously with the expression of early mitogenic genes and well before the onset of cell division. The same kinetics were observed when uninfected quiescent CHM cells were stimulated by the addition of EGF or insulin (data not shown). The level of p20K mRNA was also severely reduced when tsNY72-4RSV-infected CHM cells were transferred to the permissive temperature of 35°C. Under these conditions, pp60$^{v\text{-}src}$ was activated and CHM cells began to proliferate (3). The decline in the p20K mRNA level was gradual, and less than 10% of the p20K mRNA remained at the onset of cell division (Fig. 2B, 15 h). The repression of p20K correlates with the accumulation of mRNA of CEF-4, a transformation- and growth-regulated gene (6). To determine to what extent p20K expression is linked to quiescence, we prepared RNA from tsNY72-4RSV-infected CHM cells transferred from the permissive temperature to the nonpermissive temperature. Northern analysis indicated that under these conditions, p20K mRNA accumulation was rapid (Fig. 2C). Densitometric measurements indicated that p20K mRNA levels increased by more than 50-fold within 10 h of the temperature shift. The expression of p20K mRNA in these cells probably reflected the effects of culturing the cells in plasma, as well as contact inhibition (7). Indeed, the expression of p20K mRNA coincided with the appearance of flat, morphologically normal cells. Fully induced p20K mRNA levels preceded the reported onset of induction of genes such as that for fibronectin (14). The contrasting pattern of the CEF-4 mRNA (Fig. 2B and C) serves to illustrate the profound changes associated with the regulation of quiescence and the determination of cell proliferation or transformation by pp60$^{v\text{-}src}$.

Although CEF are not quiescent in plasma, they express the quiescence-specific polypeptides at confluence (7). In this respect, contact inhibition of CEF is equivalent to quiescence of CHM cells in plasma. This assertion is supported by the expression of the p20K transcript in contact-inhibited CEF (Fig. 3). tsNY72-4RSV-infected CEF were cultured to confluence in serum-containing medium at the nonpermissive temperature of 41°C. Transfer to the permissive temperature of 35°C and activation of pp60$^{v\text{-}src}$ resulted in transformation and, therefore, in the release from contact inhibition and the initiation of cell proliferation. Under these conditions, we observed a substantial decrease in p20K mRNA abundance, indicating that in CEF the regulation of the p20K transcript is also determined by the proliferative state of the cell (Fig. 3).

The results presented above establish an association between cell quiescence and p20K mRNA expression. Experimental data are needed to clarify the functional role, if any, of p20K in the control of cell proliferation. We also sought relevant information on the nature of p20K by sequencing the insert of clone p20K-20. Both strands of the p20K-20 insert were sequenced with enzymes and reagents purchased from U.S. Biochemical Corp. (Sequenase). Deletions were generated in the p20K-20 insert (cloned in both orientations) by limited digestion with exonuclease III (Erasabase; Promega Biotech, Madison, Wis.). Single-stranded DNAs were purified from phagemids as described by Russel et al. (21). Phagemids were produced by coinfection of *Escherichia coli* 7118 [Δ(*lac-pro*) F *lacI*^{q} *lacZ*ΔM15 *pro*$^+$ *supE*) transformed by p20K-20 or its deletion derivatives (all inserts were cloned in the SK minus bluescript vector; Stratagene) with the helper phage R408. Sequence analysis was carried out with the University of Wisconsin GCG program. The FASTP program of Lipman and Pearson (19) was used to search the protein sequence data base maintained by the National Biomedical Research Foundation, Washington,

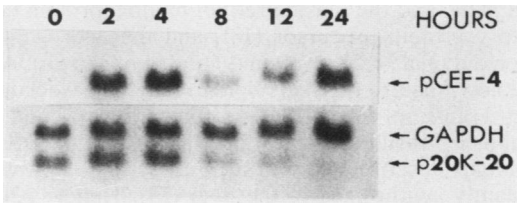

FIG. 3. Regulation of p20K mRNA in density-arrested CEF. Early passage CEF were infected with tsNY72-4RSV and cultured in Dulbecco modified Eagle medium containing 10% calf serum at the nonpermissive temperature of 41°C. At confluence, infected cells were transferred to the permissive temperature, and RNA was prepared at each of the indicated times. Total RNA was analyzed on Northern blots and probed with radioactively labeled p20K-20, pCEF-4, and GAPDH plasmid DNAs as described in the text.

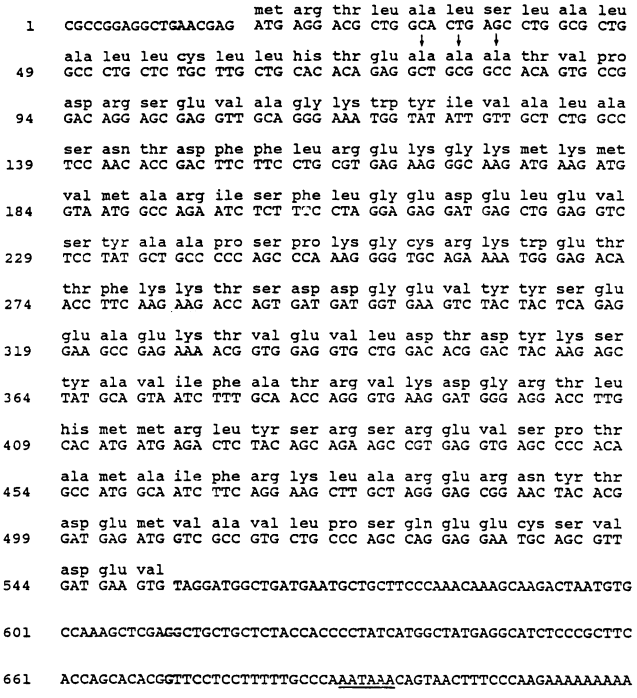

FIG. 4. Nucleotide and deduced amino acid sequences of p20K-20. The arrows indicate the predicted processing sites of the signal peptide (24). A polyadenylation sequence, AATAAA, is underlined.

D.C. (release 17). The nucleotide and predicted amino acid sequences of the longest open reading frame are presented in Fig. 4. The 178-amino-acid polypeptide is in frame with the beta-galactosidase segment provided by the vector. The initiating methionine is followed by hydrophobic residues suggestive of a signal peptide. Applications of von Heijne rules (24) predict processing at residues 20 and 21 or 22 (Ala-Ala-Ala). If processing occurs at position 21, the mature polypeptide will include 157 amino acids and will have a molecular mass of 17,980 daltons. In agreement with the isoelectric point of p20K on two-dimensional gels (7) (pI, 6.3), an overall charge of -3 is deduced from the amino acid sequence. The processed polypeptide includes two cysteines and one potential glycosylation site at residue 137 (asparagine). The hydrophobicity profile (10) of mature p20K indicates a largely hydrophobic N terminus. A computer search of the National Biomedical Research Foundation data banks indicated limited similarity with $\alpha_{2\mu}$-globulin (23) and β-lactoglobulin 1 (13) (29 and 22% sequence identities, respectively; data not shown). Limited similarities also exist between p20K and the plasma retinol-binding protein (20), the olfactory protein precursor (18), and the androgen-dependent epididymal 18.5K protein (9) (data not shown). A ligand of unknown nature (perhaps lipidic) may be associated with p20K. Transcripts of p20K are found in lung and spleen (unpublished results), suggesting that p20K is different from all proteins described above, such as $\alpha_{2\mu}$-globulin, which is abundantly synthesized in the liver (23). Hence, p20K is a novel gene product associated with cell quiescence.

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