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Epidermal growth factor or serum stimulation of rat fibroblasts induces an elevation in mRNA levels for lactate dehydrogenase and other glycolytic enzymes

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ABSTRACT

We have isolated cloned cDNAs corresponding to five mRNAs whose level is increased following stimulation of quiescent rat fibroblasts by either epidermal growth factor or serum. Partial sequencing followed by a computer search of data banks has shown that the cloned cDNAs correspond to mRNAs encoding proteins with extensive homology to lactate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, enolase, triose phosphate isomerase, and actin. The complete nucleotide sequence of a rat fibroblast lactate dehydrogenase is presented.

INTRODUCTION

The pleiotropic biochemical response of cultured cells to growth stimuli by serum or defined growth factors has been a subject of interest for many years. Many cellular responses have been documented that precede the onset of DNA replication and may therefore play a role in transferring the mitogenic stimulus to the DNA replicating machinery (see 1,2 and references therein). In addition to a general elevation in RNA and protein levels during this period, increases in the levels of specific proteins have also been described (1,2). In some cases, the function of these proteins is known; e.g. ornithine decarboxylase, the rate-limiting enzyme in polyamine biosynthesis, is elevated in G1 prior to the onset of DNA replication (3,4). Recently it has become possible to identify specific mRNAs that are elevated in response to growth stimulation (5-8). Thus Foster et al (5) have identified mRNAs containing mouse VL30 sequences that are elevated specifically by the mitogen epidermal growth factor (EGF), and Linzer and Nathans have shown that a mRNA elevated in serum or platelet-derived growth factor (PDGF)-stimulated cells is a member of the prolactin-growth hormone family (6). Expression of the oncogene c-myc in Balb/c 3T3 cells is also elevated by the growth-stimulatory effects of serum (7) or purified PDGF (8).

In an attempt to identify further mRNAs that may play an important role in the transmission of the growth signal we have isolated several cloned cDNAs corresponding to mRNAs that are more abundant in cultured rat fibroblast cells after stimulation by either serum or the mitogenic hormone epidermal growth factor. These cloned cDNAs were shown by nucleotide sequence analysis and comparison with published nucleotide or amino acid sequences to code for proteins with extensive homology to the glycolytic enzymes lactate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, enolase, and triose phosphate isomerase. In addition, another growth-induced mRNA encodes an actin.

MATERIALS AND METHODS

Cell culture

Rat-1 (9), FR3T3 (10), and MTT4 (11) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and appropriate antibiotics in a 95% air/5% CO_2 humidified atmosphere.

Epidermal growth factor

Culture grade EGF was prepared from mouse submaxillary glands and purified by BioGel P-10 chromatography as described by Savage and Cohen (12).

RNA isolation and Northern blotting

Total cytoplasmic RNA was isolated and electrophoresed on 1.5% agarose-formaldehyde gels as previously described (13). The RNA was transferred to nitrocellulose and hybridized to nick-translated DNA probes (14) as described (15) except that dextran sulfate was present at 4.0%.

Cloning of double-stranded cDNA

The original library of 300 cDNA clones was prepared from RNA isolated from FR3T3-derived MTT4 cells (11) using PstI-linearized pBR322 and the G-C tailing technique and was generously provided by P. Masiakowski and N. Glaichenhaus. Differential screening was carried out using colony arrays on Whatman 540 paper as previously described (13). A second library was constructed from RNA from the same cells as described by Breathnach and Harris (16) in order to isolate full-length cDNAs and was screened by hybridization to the nick-translated fragments from the pBR322 clones of interest.

Primer-extension

A plasmid (pLDH-3) was made by introducing a 130bp EcoRI-PvuII fragment of pLDH-2 (see Figure 2) between the EcoRI and PvuII sites of pBR322.

The primer was prepared by 5'-end-labelling an AvaII digest of pLDH-3 with γ-[^{32}P]-ATP and T4 polynucleotide kinase, cleaving with HhaI, and isolating the labeled strand of a 53 nucleotide AvaII-HhaI fragment from a denaturing 8% sequencing gel. The primer was hybridized with the RNA samples described in the legend to Figure 4 using approximately 1 pmole of primer per 100 μg of RNA at 42°C for 2.5h. Conditions for the hybridization and extension procedure were as described previously (17). The RNA was digested for 1h at 65°C in 50mM NaOH, and the reaction mixture neutralized and precipitated as described (18). The DNA products were electrophoresed on a 8% sequencing gel.

DNA sequencing

Portions of the DNA sequence were derived using the method of Maxam and Gilbert (19). Sequences were also obtained using modifications of the dideoxy sequencing technique (20, 21) and M13 derivatives (22, 23). Deoxyadenosine 5' (α-[^{35}S] thio)triphosphate was used as the radioactive label and the sequences were read from a buffer gradient gel as described (24).

Computer search programs

The National Biomedical Research Foundation (NBRF, "Dayhoff") database was searched using either the Wilbur and Lipman program (25) which was run at the Imperial Cancer Research Fund in London, England, or by the protein data bank search program which was run via network on the computer service at the Centre Interuniversitaire de Traitement de l'Information (C.I.T.I.2), in Paris, France. The European Molecular Biology Laboratory (EMBL) data bank of nucleotide sequences was searched using a batch homology search program (26).

RESULTS

Isolation of cloned cDNAs for growth-induced mRNAs

To identify mRNAs whose levels are elevated under conditions of cellular growth, Rat-1 fibroblast cells were arrested by serum starvation, followed by overnight stimulation with 10ng/ml of the polypeptide mitogen epidermal growth factor (EGF) in serum-free medium. PolyA + RNA isolated from cells with or without EGF stimulation was used to prepare cDNA probes for hybridization to duplicate filters of a plasmid library of 300 cDNA clones originally made for another purpose and prepared using RNA from a rat fibroblast line derived from FR3T3 cells [line MTT4 (11)]. Of these 300 clones, 14 demonstrated an approximately 5-fold elevation in the intensity of the hybridization signal with the EGF-stimulated sample (data not

shown). Cross-hybridization experiments allowed plasmids from these clones to be grouped into five different families. The plasmid with the largest insert for each family was chosen for further study. The plasmids were named pLDH, pGAPDH, pTIM, pENO and pACT. Sizes of inserts are 1.6, 0.5, 0.4, 0.6 and 0.6 kbp respectively. The pLDH family consisted of 2 members, pGAPDH of 7 members, pTIM of 1 member, pENO of 3 members and pACT of 1 member.

Timecourse of induction of mRNA species by cellular growth

To characterize the induction of the mRNAs corresponding to the plasmids described above by growth stimulation, cytoplasmic RNA was isolated from rat fibroblast cells that were stimulated from a resting to a growing state by one of two different protocols : a) confluent, density-arrested FR3T3 cells were stimulated by the addition of fresh medium containing 10% fetal calf serum, and b) Rat-1 fibroblasts were serum-starved for 48h, followed by the addition of 20ng/ml of EGF. At times between 0 and 24h after stimulation with EGF or serum, the cells were harvested and total cytoplasmic RNA isolated as described (13). The RNA (10μg) was electrophoresed on a formaldehyde-agarose gel, blotted onto nitrocellulose, and hybridized with nick-translated plasmid probes. The results are shown in Figure 1.

The hybridization patterns observed reflect an elevation in the levels of the mRNAs corresponding to pLDH, pGAPDH, pENO, pTIM, and pACT following stimulation of growth by either protocol. However, although serum stimulation of Rat-1 cells results in an elevation of the levels of these same RNAs, EGF stimulation of FR3T3 cells using the protocol employed with the Rat-1 cells has no such effect (data not shown). In the case of pLDH, the hybridization signal is barely detectable in density-arrested FR3T3 cells (lane 0, left panel), but gradually increases with time to levels 12-fold higher after 24h of serum stimulation. This level is roughly equivalent to the level of pLDH-hybridizing mRNA found in exponentially growing FR3T3 cells (lane X). Similar, though less dramatic, results are found following stimulation of serum-starved Rat-1 fibroblasts with EGF. pLDH-hybridizing mRNA is detectable in arrested cells (lane 0, right panel) and increases about 3-fold after 24h of EGF stimulation to levels equivalent to those in exponentially growing Rat-1 cells (lane X). The decreased magnitude of the response in Rat-1 cells relative to FR3T3 cells is partly accounted for by higher levels of pLDH-hybridizing mRNA in resting Rat-1 cells relative to the FR3T3 cells (compare lane 0, right and

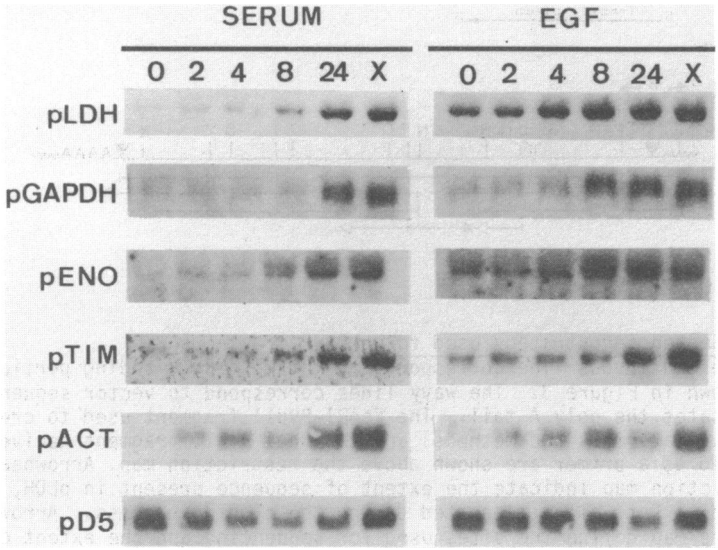


Fig. 1. Timecourse of induction of mRNA species by serum and EGF. Autoradiographs of Northern blots of RNA isolated from quiescent FR3T3 cells stimulated by the addition of 10% fetal calf serum and hybridized with the indicated nick-translated probes are shown in the left panels. The right panels represent similar samples from serum-starved Rat-1 cells stimulated with 20 ng/ml EGF in serum free medium. The numbers above each lane refer to the time in hours of exposure to serum or EGF. X=RNA samples isolated from cells growing exponentially in serum-containing medium.

left panels), and therefore cannot be interpreted solely as a result of a weaker induction by EGF as compared to serum. In both protocols, the increase in pLDH-hybridizing mRNA becomes evident after 4h of growth stimulation and is marked 8h after stimulation.

A pattern of induction very similar to that of the pLDH-hybridizing mRNA was observed with the mRNAs corresponding to the plasmids pGAPDH, pENO, and pTIM (Figure 1). The results obtained with pACT differed from the others by respect of the very rapid increase in mRNA levels following serum or EGF stimulation (elevated by 2h, lane 2, both panels). Approximate sizes of the RNA species hybridizing to pLDH, pGAPDH, pENO, pTIM, and pACT are 1.8, 1.5, 2.0, 1.4 and 1.9 kb respectively, determined using rat and E. coli ribosomal RNAs as size markers.

As a control, we chose a plasmid (pD5) which did not demonstrate any differential hybridization in the original screening. The levels of pD5-hybridizing mRNA remained roughly constant following growth stimulation

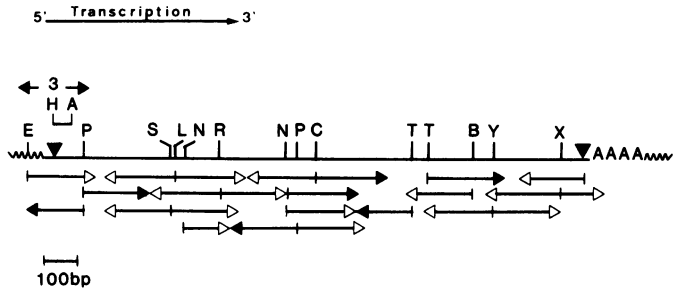


Fig. 2. Sequencing strategy used for pLDH-2.
The continuous line corresponds to the messenger-coding portion of pLDH-2 shown in Figure 3. The wavy lines correspond to vector sequences. AAAA indicates the poly A tail. The EcoRI-PvuII fragment used to create pLDH-3 (see Materials and Methods) and the HhaI-AvaII fragment derived from it and used as a primer are shown above the restriction map. Arrowheads on the restriction map indicate the extent of sequence present in pLDH, and correspond to PstI sites generated during the cloning process. Arrows beneath the map define the sites used for sequencing and the extent of sequence read. Arrows ending in filled in or open arrowheads represent sequences determined by the dideoxy or Maxam and Gilbert technique respectively. E=EcoRI; P=PvuII; S=SacII; L=ApaI; N=NaeI; R=EcoRV; C=HincII; T=PstI; B=BstEII; Y=SphI; X=BglII; H=HhaI; A=AvaII. Only the HhaI and AvaII sites used to make the primer are shown.

by either protocol (Figure 1). Furthermore, levels of RNAs complementary to three other randomly chosen plasmids were unchanged following serum stimulation of quiescent FR3T3 cells (not shown). The behavior of these RNAs and the RNA complementary to pD5 clearly did not mimic that of the other RNAs shown in Figure 1.

Isolation of plasmids carrying large cDNA inserts

In the hope of identifying the mRNA species induced by cellular growth, we decided to sequence the corresponding cloned cDNAs for comparison with published nucleotide or amino acid sequences. The cDNA inserts carried by the original plasmids were however relatively short. A new cDNA library was therefore prepared from FR3T3 cell RNA using the Okayama and Berg technique (27) as described by Breathnach and Harris (16). This method provides clones with a greater retention of sequences representing the 5' ends of mRNAs due to the elimination of the use of S1 nuclease. This library was screened with nick-translated inserts from the original plasmids, and a new series of plasmids isolated. The plasmids with the largest inserts (referred to as pLDH-2, pENO-2, pGAPDH-2, pTIM-2, and pACT-2) were used for the work described below. The size of the cDNA inserts

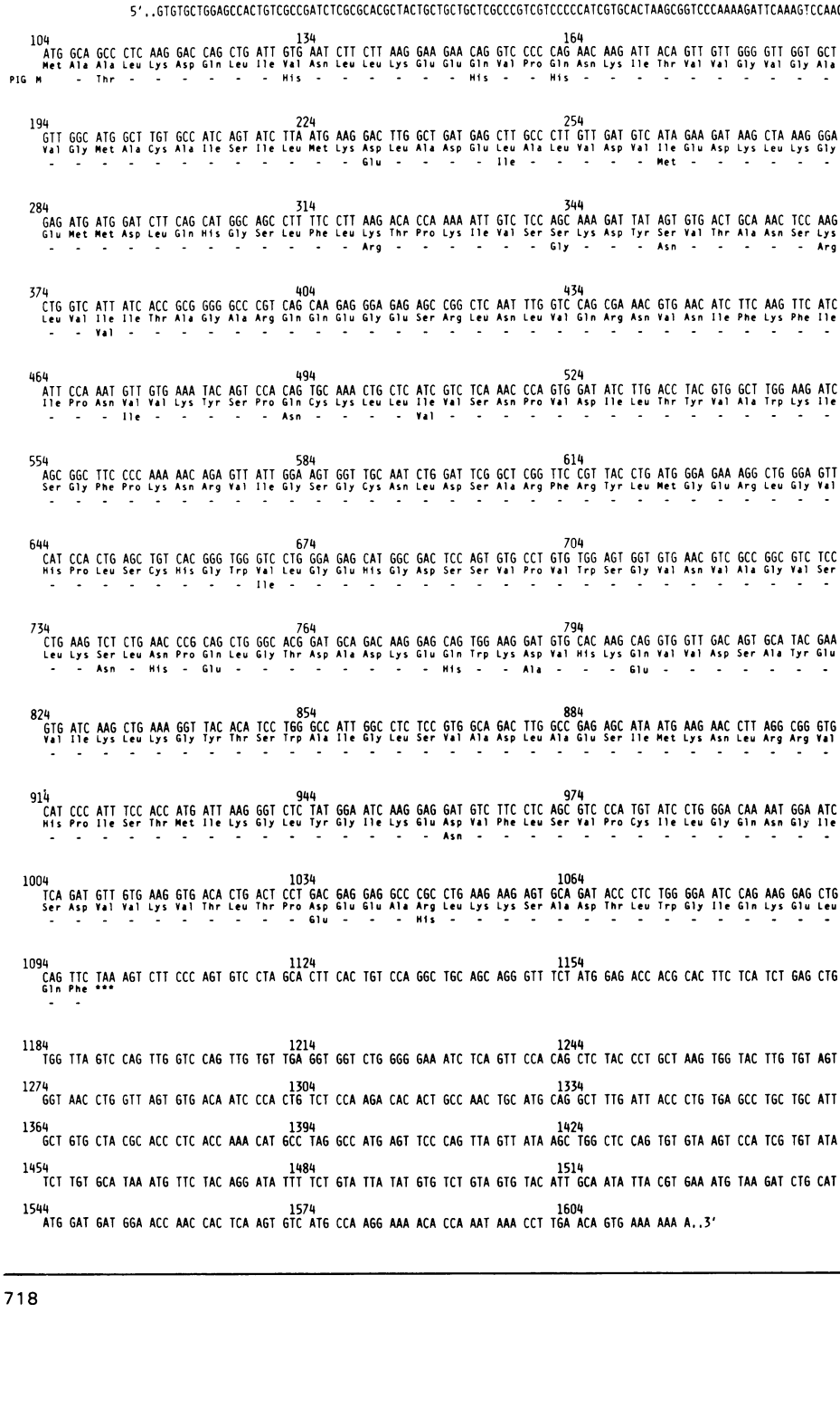
carried by pLDH-2, pENO-2, pGAPDH-2, and pTIM-2 (1.8, 1.9, 1.4 and 1.3 kbp respectively) suggests that these plasmids may carry full size cDNA copies of the corresponding mRNAs. The cDNA insert of pACT-2 (1.4kb) is however clearly shorter than the corresponding mRNA (~1.9kb).

pLDH-2 contains a full-length lactate dehydrogenase cDNA

We chose to begin our studies with pLDH-2. The cDNA insert in pLDH-2 was sequenced using both the Maxam and Gilbert and the dideoxy methods. Sequencing strategies are detailed in Figure 2. The sequence obtained indicates that pLDH-2 contains a 1609 bp cDNA insert (Figure 3). The putative initiation codon ATG (corresponding to a mRNA AUG sequence) occurs 104bp from the 5' end of the insert. Starting with this codon, there is an open reading frame that extends to residue 1099.

The pLDH mRNA thus codes for a 331 amino acid protein, not including the initiating methionine residue. No homologous sequence was found by a computer search of the nucleotide sequences stored in the EMBL data-bank. However, comparison to the protein sequences stored in the National Biomedical Research Foundation (NBRF, "Dayhoff") database showed that the pLDH-2 mRNA codes for a protein with 92% homology to porcine lactate dehydrogenase (LDH) M (muscle) chain (28). A comparison of these two sequences shown in Figure 3 reveals 25 amino acids which differ, 15 of which are substitutions by amino acids retaining similar charge or polarity properties. Of the remaining 10 amino acid substitutions, 7 involve amino acids with "borderline" properties (29) and 3 are glu-gln or asp-asn substitutions which are the result of single nucleotide changes. In addition, the sequence also shows 74% homology with porcine LDH H (heart) chain (28) and 86% homology with the M chain LDH from chicken (30). As the lactate dehydrogenase enzymes of the M, H, or C (testes) types from rat have not been sequenced, it is not possible to identify definitively the sequence. However it is clear that pLDH-2 represents an mRNA coding for a rat lactate dehydrogenase protein chain, and that the chain is most probably of the M type as expected in cells of fibroblast origin.

To determine if the entire 5' end sequences of the LDH mRNA were present in pLDH-2, a 53bp HhaI-AvaII fragment (complementary to residues 30 to 83, see Figure 2 and 3) was used for primer extension studies (see Materials and Methods for details). The primer, 5'-terminally labeled at the AvaII end, was hybridized with poly A^+ RNA isolated from growing FR3T3-derived cells and elongated using reverse transcriptase. Following digestion of the RNA with NaOH, the elongated DNA was analyzed on a denaturing


5',.GTGTGCTGGAGCCACTGTCGCCGATCTCGCGCACGCTACTGCTGCTGCTCGCCCGTCGTCCCCCATCGTGCACTAAGCGGTCCCAAAAGATTCAAAGTCCAAG
104 134 164
ATG GCA GCC CTC AAG GAC CAG CTG ATT GTG AAT CTT CTT AAG GAA GAA CAG GTC CCC CAG AAC AAG ATT ACA GTT GTT GGG GTT GGT GCT
Met Ala Ala Leu Lys Asp Gln Leu Ile Val Asn Leu Leu Lys Glu Glu Gln Val Pro Gln Asn Lys Ile Thr Val Val Gly Val Gly Ala
PIG M - Thr - - - - - - His - - - - - - - His - - His - - - - - - - - -
194 224 254
GTT GGC ATG GCT TGT GCC ATC AGT ATC TTA ATG AAG GAC TTG GCT GAT GAG CTT GCC CTT GTT GAT GTC ATA GAA GAT AAG CTA AAG GGA
Val Gly Met Ala Cys Ala Ile Ser Ile Leu Met Lys Asp Leu Ala Asp Glu Leu Ala Leu Val Asp Val Ile Glu Asp Lys Leu Lys Gly
- - - - - - - - - - - - Glu - - - - Ile - - - - - - Met - - - - - -
284 314 344
GAG ATG ATG GAT CTT CAG CAT GGC AGC CTT TTC CTT AAG ACA CCA AAA ATT GTC TCC AGC AAA GAT TAT AGT GTG ACT GCA AAC TCC AAG
Glu Met Met Asp Leu Gln His Gly Ser Leu Phe Leu Lys Thr Pro Lys Ile Val Ser Ser Lys Asp Tyr Ser Val Thr Ala Asn Ser Lys
- - - - - - - - - - - - Arg - - - - - - Gly - - - Asn - - - - - Arg
374 404 434
CTG GTC ATT ATC ACC GCG GGG GCC CGT CAG CAA GAG GGA GAG AGC CGG CTC AAT TTG GTC CAG CGA AAC GTG AAC ATC TTC AAG TTC ATC
Leu Val Ile Ile Thr Ala Gly Ala Arg Gln Gln Glu Gly Glu Ser Arg Leu Asn Leu Val Gln Arg Asn Val Asn Ile Phe Lys Phe Ile
- - Val -
464 494 524
ATT CCA AAT GTT GTG AAA TAC AGT CCA CAG TGC AAA CTG CTC ATC GTC TCA AAC CCA GTG GAT ATC TTG ACC TAC GTG GCT TGG AAG ATC
Ile Pro Asn Val Val Lys Tyr Ser Pro Gln Cys Lys Leu Leu Ile Val Ser Asn Pro Val Asp Ile Leu Thr Tyr Val Ala Trp Lys Ile
- - - Ile - - - - - Asn - - - - Val - - - - - - - - - - - - - - -
554 584 614
AGC GGC TTC CCC AAA AAC AGA GTT ATT GGA AGT GGT TGC AAT CTG GAT TCG GCT CGG TTC CGT TAC CTG ATG GGA GAA AGG CTG GGA GTT
Ser Gly Phe Pro Lys Asn Arg Val Ile Gly Ser Gly Cys Asn Leu Asp Ser Ala Arg Phe Arg Tyr Leu Met Gly Glu Arg Leu Gly Val
- -
644 674 704
CAT CCA CTG AGC TGT CAC GGG TGG GTC CTG GGA GAG CAT GGC GAC TCC AGT GTG CCT GTG TGG AGT GGT GTG AAC GTC GCC GGC GTC TCC
His Pro Leu Ser Cys His Gly Trp Val Leu Gly Glu His Gly Asp Ser Ser Val Pro Val Trp Ser Gly Val Asn Val Ala Gly Val Ser
- - - - - - - - - Ile -
734 764 794
CTG AAG TCT CTG AAC CCG CAG CTG GGC ACG GAT GCA GAC AAG GAG CAG TGG AAG GAT GTG CAC AAG CAG GTG GTT GAC AGT GCA TAC GAA
Leu Lys Ser Leu Asn Pro Gln Leu Gly Thr Asp Ala Asp Lys Glu Gln Trp Lys Asp Val His Lys Gln Val Val Asp Ser Ala Tyr Glu
- - Asn - His - Glu - - - - - - - - - His - - Ala - - - Glu - - - - - - - -
824 854 884
GTG ATC AAG CTG AAA GGT TAC ACA TCC TGG GCC ATT GGC CTC TCC GTG GCA GAC TTG GCC GAG AGC ATA ATG AAG AAC CTT AGG CGG GTG
Val Ile Lys Leu Lys Gly Tyr Thr Ser Trp Ala Ile Gly Leu Ser Val Ala Asp Leu Ala Glu Ser Ile Met Lys Asn Leu Arg Arg Val
- -
914 944 974
CAT CCC ATT TCC ACC ATG ATT AAG GGT CTC TAT GGA ATC AAG GAG GAT GTC TTC CTC AGC GTC CCA TGT ATC CTG GGA CAA AAT GGA ATC
His Pro Ile Ser Thr Met Ile Lys Gly Leu Tyr Gly Ile Lys Glu Asp Val Phe Leu Ser Val Pro Cys Ile Leu Gly Gln Asn Gly Ile
- - - - - - - - - - - - - - - - Asn - - - - - - - - - - - - - -
1004 1034 1064
TCA GAT GTT GTG AAG GTG ACA CTG ACT CCT GAC GAG GAG GCC CGC CTG AAG AAG AGT GCA GAT ACC CTC TGG GGA ATC CAG AAG GAG CTG
Ser Asp Val Val Lys Val Thr Leu Thr Pro Asp Glu Glu Ala Arg Leu Lys Lys Ser Ala Asp Thr Leu Trp Gly Ile Gln Lys Glu Leu
- - - - - - - - - - Glu - - - His - - - - - - - - - - - - - - -
1094 1124 1154
CAG TTC TAA AGT CTT CCC AGT GTC CTA GCA CTT CAC TGT CCA GGC TGC AGC AGG GTT TCT ATG GAG ACC ACG CAC TTC TCA TCT GAG CTG
Gln Phe ***
- -
1184 1214 1244
TGG TTA GTC CAG TTG GTC CAG TTG TGT TGA GGT GGT CTG GGG GAA ATC TCA GTT CCA CAG CTC TAC CCT GCT AAG TGG TAC TTG TGT AGT
1274 1304 1334
GGT AAC CTG GTT AGT GTG ACA ATC CCA CTG TCT CCA AGA CAC ACT GCC AAC TGC ATG CAG GCT TTG ATT ACC CTG TGA GCC TGC TGC ATT
1364 1394 1424
GCT GTG CTA CGC ACC CTC ACC AAA CAT GCC TAG GCC ATG AGT TCC CAG TTA GTT ATA AGC TGG CTC CAG TGT GTA AGT CCA TCG TGT ATA
1454 1484 1514
TCT TGT GCA TAA ATG TTC TAC AGG ATA TTT TCT GTA TTA TAT GTG TCT GTA GTG TAC ATT GCA ATA TTA CGT GAA ATG TAA GAT CTG CAT
1544 1574 1604
ATG GAT GAT GGA ACC AAC CAC TCA AGT GTC ATG CCA AGG AAA ACA CCA AAT AAA CCT TGA ACA GTG AAA AAA A,.3'

urea -polyacrylamide gel. The results are shown in Figure 4, lane c. A number of bands are observed. 1). Residual 53-nucleotide primer migrates as expected in relation to size markers and a sample of non-extended primer (Figure 4, lane a). 2). A strong band is observed migrating with an apparent size of 64 nucleotides. However, this band is present even when the reaction is run in the absence of mRNA (see Figure 4, lane d), suggesting that it results from self-priming. Indeed, examination of the nucleotide sequence of the primer indicates a 6 base sequence GCGTGC at its 3' -end which can loop back and base pair with the sequence GCACGT close to its 5'-end (see underlined sequences : 3'...GCGTGCGATGACGACGACGAGCGGGCAGCAGGGGGTAGCACGTGATTCGCCAG...5'. This allows an 11 base elongation of the primer by self-priming, thus forming the 64 nucleotide species observed. In addition, several faint bands between 53 and 60 nucleotides in size visible in lane c are also visible in lane d, and therefore are unrelated to mRNA elongation. 3) A band of size ~83 nucleotides is observed. This band is only visible when extension is carried out in the presence of poly A^+ RNA and thus reflects true primer extension on a mRNA template [band present in lane c, absent in lane d ; however, a very faint band may be seen on the original autoradiogram when total RNA is used as a template (lane a)]. Subtraction of the size of the primer (53 nucleotides) results in the conclusion that the primer was extended 30 nucleotides beyond the HhaI site at its 3'-end, a figure which corresponds to the number of nucleotides present in pLDH-2 upstream of the HhaI site (see Figure 3). A faint band with a size of approximately 84 nucleotides is also specific to lane c, indicating that there could be heterogeneity in the mRNA start site for this transcript. The pLDH-2 clone thus appears to contain the entire LDH mRNA, within the resolution of the techniques employed. The mRNA has a 103 nucleotide 5'-non-translated region and a 510 nucleotide 3'-untranslated region. This latter region carries the classic polyadenylation signal AAUAAA (31) 18 nucleotides before the poly A tail (Figure 3).

Fig. 3. Sequence of lactate dehydrogenase mRNA as deduced from the sequence of pLDH-2.
The sequence is shown as DNA. The amino acid sequence deduced from this DNA sequence is shown below it. Also shown for comparison is the sequence of a pig lactate dehydrogenase (M type). Where the two protein sequences are identical this has been indicated by a dash. Where the sequences differ, the residue in the porcine enzyme has been indicated.

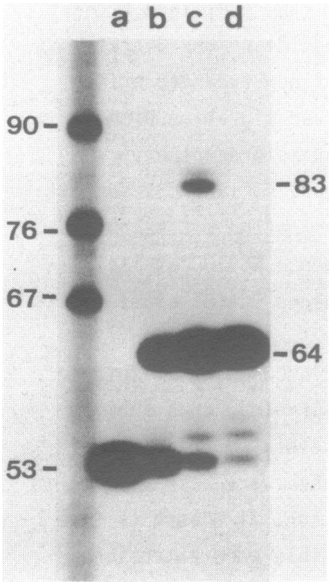


Fig. 4. Primer extension of pLDH-2. Primer extension studies were carried out in the presence of 100 μg of total cytoplasmic RNA isolated from MTT4 cells (lane b) or approximately 10 μg of polyA+ RNA isolated from total cytoplasmic MTT4 RNA by oligo dT-cellulose chromatography (lane c). The reaction mixture represented in lane d contained 10 μg of E.coli tRNA. Lane a = primer alone. Size markers are ^{32}P-labeled MspI fragments from pBR322.

Partial nucleotide sequences and identification of growth-induced cDNA clones

The successful identification of the pLDH mRNA prompted us to attempt the identification of the remaining growth induced mRNAs by partial sequencing of the corresponding cloned cDNAs. Sequencing strategies are shown in Figure 5. The resulting nucleotide sequences were either compared directly to sequences in the EMBL data bank, or the deduced protein sequences were compared with sequences in the NBRF protein data bank. The partial nucleotide sequences and deduced amino acid sequences obtained for

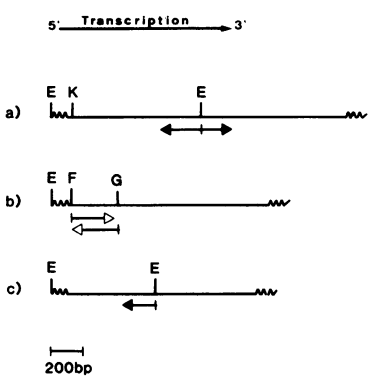


Fig. 5. Sequencing strategy for pENO-2 (a), pGAPDH-2 (b) and pTIM-2 (c). Symbols are as in the legend to Figure 2. In addition, K=KpnI; F=XbaI; G=NcoI. The sizes of the cDNA inserts shown include the poly A tail. The exact extent of the messenger coding sequence thus cannot be defined. pACT-2 was sequenced from an EcoRI site present in vector sequences close to the 5'-end of the cDNA insert by the Maxam and Gilbert technique.

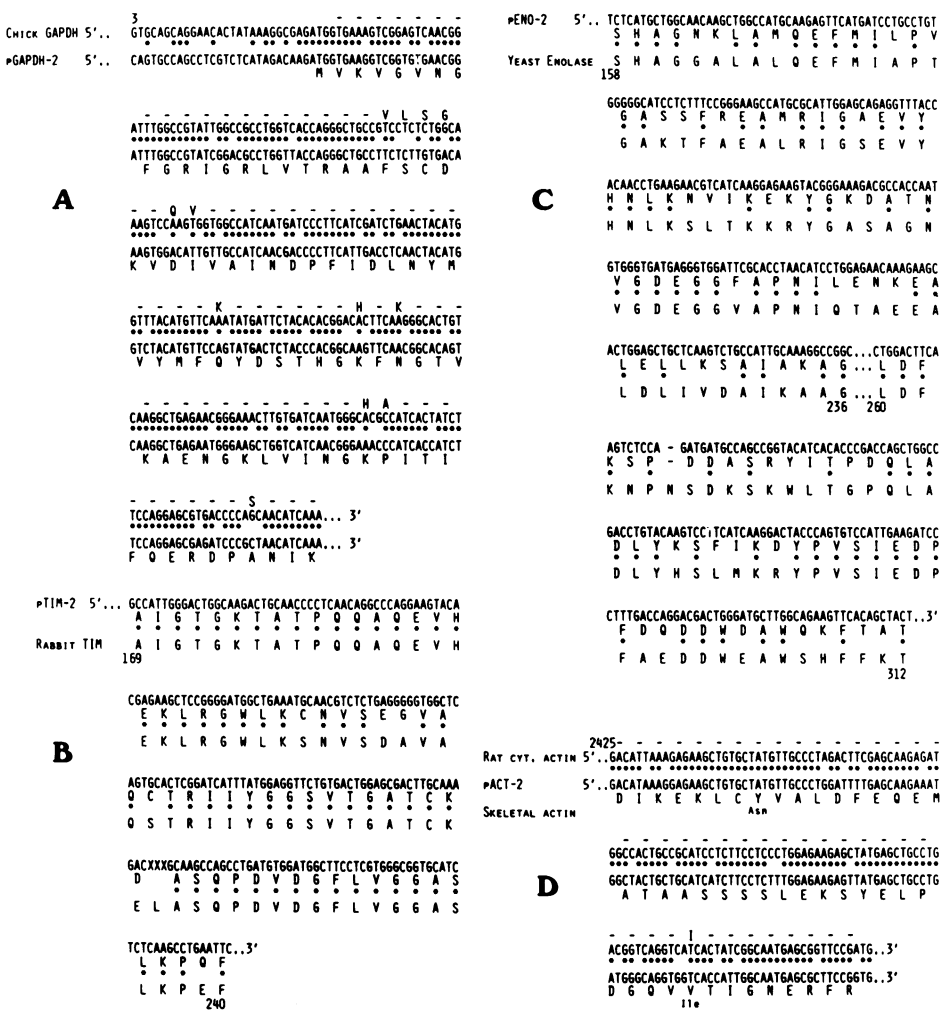


Fig. 6. Partial sequence of pGAPDH-2, pTIM-2, pENO-2 and pACT-2 and comparison with published sequences.

The amino acid sequence deduced from the DNA sequence is shown below it. Panel A : pGAPDH-2 sequence compared to chicken glyceraldehyde 3-phosphate dehydrogenase nucleotide and protein sequence (44). Panel B : pTIM-2 sequence compared to rabbit triose phosphate isomerase amino acid sequence (45). Panel C : pENO-2 sequence compared to yeast enolase amino acid sequence (46). Panel D : pACT-2 sequence compared to rat cytoplasmic β-actin nucleotide and amino acid sequence (33) and rat skeletal muscle actin protein sequence (32). Where two compared nucleotide sequences are identical this is indicated with a star. Where two compared protein sequences are identical, this is indicated with either a star (panel B and C), a dash (panel A and D), or the lack of an alternate residue (panel D, for rat skeletal muscle comparison).

Table I. Homology of DNA and protein sequences.
The percent homology between DNA and protein sequences reported in Figure 6 is shown.

Plasmid	homologous to	% homology DNA sequence	% homology protein sequence	Data bank screened
pGAPDH-2	chicken glyceraldehyde phosphate dehydrogenase	77	85	EMBL
pTIM-2	rabbit triose phosphate isomerase	-	90	NBRF
pENO-2	yeast enolase	-	62	NBRF
pACT-2	rat cytoplasmic β-actin	86	98	EMBL

clones pGAPDH-2, pENO-2, pTIM-2, and pACT-2 are shown in Figure 6, together with corresponding data for the genes or proteins to which they are most homologous. The data shown in Figure 6 and analyzed in Table 1 points strongly toward the conclusion that pGAPDH-2, pENO-2, and pTIM-2 correspond to cDNA clones for rat glyceraldehyde 3-phosphate dehydrogenase, enolase, and triose phosphate isomerase respectively, when allowance is made for some inter-species variation. (The sequence of none of the rat glycolytic enzymes is known). The 62% homology of the pENO-2 amino acid sequence with yeast enolase is not an unexpected homology value between the enolases of the two species, since when the sequences of chicken GAPDH or rabbit TIM shown in Figure 6 are compared to the corresponding regions of the yeast enzymes, less than 60% of the residues are homologous. From the data shown in Figure 6, we conclude that pGAPDH-2 contains the initiation codon for the corresponding protein, and thus should harbor a complete coding sequence. A complete coding sequence is also carried by pENO-2 (date not shown).

It is clear that pACT-2 corresponds to an actin. However, the nucleotide sequence of pACT-2 differs from that of a rat skeletal muscle actin gene (32) and a rat cytoplasmic β-actin gene (33). Thus we cannot at present define the type of actin encoded by pACT-2.

DISCUSSION

The examination of specific mRNAs that are elevated in response to growth stimulation may provide a clue to the biochemical events that regulate cellular growth. Thus studies on mRNAs induced by serum (6,34) or

the growth factors PDGF (8,35) and EGF (5-8,36,37) have been reported. In some cases the identity of the induced mRNAs is known; eg EGF stimulation of appropriate cells induces mRNA levels for prolactin (36,37), VL30 (5), c-myc (7) and a prolactin-like growth hormone (6). We show here that EGF treatment of quiescent Rat-1 cells results in an accumulation of mRNAs coding for four glycolytic enzymes (lactate dehydrogenase, triose phosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase and enolase) and an actin. Serum stimulation of another rat fibroblast line (FR3T3) has the same effect, although in this case EGF alone is incapable of eliciting this response.

As the library used for the differential screening experiments described here consisted of only 300 clones, it is clear that we have identified only a sample of mRNAs induced by the growth response. However, we have recently screened an additional 2000 clones from a rat fibroblast cDNA library wihout identifying any other EGF-induced clones. Nonetheless, it is worth bearing in mind that mRNAs present in relatively low levels are very difficult to detect using cDNA probes prepared from total mRNA populations. Indeed, Maniatis et al. (18) estimate the limit of detection to be 0.5%.

It is not clear if the elevation in the levels of these mRNAs, and presumably of the corresponding proteins, is directly related to the triggering of the mitogenic signal, or if it is a consequence of the general growth response. However, it is tempting to speculate on a possible role for an increase in the levels of these mRNAs in the proliferative response. It has been hypothesized that activation of glycolysis is a primary effect of growth-promoting agents (38), and may indeed be the ultimate regulatory process for the initiation of DNA synthesis (39). Previous work has highlighted the importance of rapid post-translational modification of existing enzyme molecules. In this respect it is interesting that Cooper et al (40) have shown that the glycolytic enzymes enolase, phosphoglycerate mutase and lactate dehydrogenase are phosphorylated at tyrosine in cells transformed by Rous sarcoma virus, a virus whose transforming protein possesses tyrosine kinase activity. Such a modification might alter the activity of the enzymes, either alone or in a multi-enzyme complex, and thus contribute to the altered growth control evident in these cells. However, it is clear that an elevation of mRNA levels may represent a necessary complementary means of increasing enzyme activity which might be of greater importance at a later stage of the growth response. The availability of cDNA clones for

four rat glycolytic enzymes should allow us to investigate this possibility.

Recent results indicate that lactate dehydrogenase is homologous to a rat liver helix-destabilizing protein, and indeed, that rat liver lactate dehydrogenase is a DNA-binding protein associated with transcriptionally-active regions of chromatin (41). It is possible that this previously unknown activity of lactate dehydrogenase might be important during DNA replication. Perhaps other glycolytic enzymes may possess activities with a direct relevance to growth control.

The rapid elevation of an actin mRNA following growth stimulation is also intriguing. As we have shown, the cloned cDNA is similar, but clearly different, to the rat cytoplasmic β-actin described by Nudel et al (33). In light of the rapid induction of this mRNA following growth stimulation (see Figure 1), and the recent observation that the 43-kd STF transcription factor has striking similarities to actin (42), it is tempting to speculate that a role exists for our actin in the regulation of cellular growth by means of its ability to stimulate gene transcription. Also of interest in this respect is the discovery that a portion of an oncogene from the Gardner-Rasheed feline sarcoma virus codes for a protein with extensive homology to actins (43).

The isolation of full-length cDNA clones for the rat glycolytic enzymes lactate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, enolase, and triose phosphate isomerase opens the way for further studies on the regulation of these enzymes during the process of cell growth and DNA synthesis in normal and tumorigenic cells. In addition, extensive studies on the mechanism of action of these enzymes have been carried out. The availability of cloned cDNAs carrying entire amino acid coding sequences will allow specific mutated proteins to be made and the extension of these studies toward a complete structure-function analysis of the corresponding enzymes.

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