Cyclic AMP Regulation of Lactate Dehydrogenase

We have cloned DNA complementary to mRNA coding for rat C6 glioma cell lactate dehydrogenase M-subunit. Double-stranded DNA complementary to a portion of lactate dehydrogenase mRNA was inserted into the Pst I site of plasmid pBR322 by the dC-dG tailing technique and amplified in Escherichia coli HB101. A recombinant plasmid containing lactate dehydrogenase cDNA was identified by colony hybridization to a cDNA probe prepared from partially purified lactate dehydrogenase mRNA and by hybridization-selected translation. The recombinant plasmid (pRLD42) contains a 680 nucleotide insert of lactate dehydrogenase mRNA.

Hybridization of nick-translated pRLD42 to glioma cell poly(A)^+RNA separated on agarose gel and transferred to nitrocellulose exhibited $M_0 = 5.9 \times 10^6$ for lactate dehydrogenase mRNA. Furthermore, Northern blot analysis of RNA from unstimulated and isoproterenol-stimulated glioma cells indicated a 2-fold increase of lactate dehydrogenase mRNA molecules in stimulated cells. The 2-fold increase of lactate dehydrogenase mRNA was confirmed by RNA-excess kinetic hybridization using pRLD42 DNA and poly(A)^+RNA from unstimulated, isoproterenol-, and dibutyryl cAMP-stimulated glioma cells. These data demonstrate that isoproterenol and dibutyryl cAMP cause an increase of the number of lactate dehydrogenase M-subunit mRNA molecules in glioma cells which, in part, determines the extent of synthesis of the lactate dehydrogenase M-subunit.

The control of protein synthesis by cyclic AMP has been the topic of extensive research in recent years (1, 2). Although there is conclusive evidence in prokaryotes for a direct effect of cAMP on gene transcription (for review, see Ref. 3), the complexity of eukaryotic systems has, to date, prevented the elucidation of the mechanism underlying cAMP control of eukaryotic protein synthesis. There is evidence that cAMP modulates the de novo synthesis of specific proteins rather than the regulation of the abundance of proteins by modification of protein degradation (1, 2, 4-11). Recent results have suggested that cAMP modulation of the synthesis of several eukaryotic proteins may be due to an accumulation of specific mRNA (9-13). Indirect quantitation through in vitro translation has shown that cAMP-mediated increases in the functional levels of mRNA for tyrosine aminotransferase (12, 13), phosphoenolpyruvate carboxykinase (10), and lactate dehydrogenase M-subunit (11). Furthermore, Brown and Papaconstantinou (9), using a cDNA probe for mouse albumin, demonstrated that dibutyryl cAMP is capable of increasing the number of molecules of albumin mRNA in the Hepa-2 mouse hepatoma cell line. While these findings indicate a regulation by cAMP of the levels of mRNA of the proteins studied, there is no evidence which would allow a determination whether cAMP acts through regulation of transcriptional and/or post-transcriptional events. Several investigators (1, 14-16), using inhibitors of protein and RNA synthesis, have come to the conclusion that cAMP acts at a post-transcriptional level, conceivably through stimulation of protein chain elongation (17). While it is possible that cAMP may regulate different enzymes in a specific way and may have diverse points of action which include transcriptional and/or post-transcriptional regulatory events, additional studies are required to clarify these questions.

Our laboratory has recently begun a systematic investigation of the cAMP-mediated induction of lactate dehydrogenase in the rat C6 glioma cell line. This model offers the advantages that one can study the regulation of synthesis of a well-characterized protein under conditions that can be rigorously controlled. In addition, lactate dehydrogenase represents a substantial portion of the total protein and mRNA activity in rat C6 glioma cells, thus facilitating quantitative studies and purification of lactate dehydrogenase and its mRNA. We have previously demonstrated that the isoproterenol- or dibutyryl cAMP-mediated induction of lactate dehydrogenase activity in the rat C6 glioma cells is preceded by specific activation of cytoplasmic and nuclear cAMP-dependent protein kinase (18), phosphorylative modification of several histones (19), and involves an increased de novo synthesis of the lactate dehydrogenase M-type subunit and increased functional levels of lactate dehydrogenase mRNA (11). In order to more clearly define the mechanism of regulation of lactate dehydrogenase gene expression, we have developed a hybridization probe for lactate dehydrogenase mRNA. We report here our use of a partially purified lactate dehydrogenase mRNA probe for lactate dehydrogenase mRNA in glioma cells.

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¶ The abbreviations used are: cAMP, adenosine 3',5'-monophosphate; dibutyryl cAMP, N(6),O(2')-dibutyryl adenosine 3',5'-monophosphate; SDS, sodium dodecyl sulfate; NaCl/P, phosphate-buffered saline; Poly(A)^+RNA, RNA containing a polyadenylic acid sequence; dscDNA, double-stranded complementary DNA; kbp, kilobase pair; bp, base pair.
to construct and select a cloned recombinant plasmid DNA containing a portion of the lactate dehydrogenase mRNA sequence. Furthermore, we have utilized this plasmid for the quantitation of lactate dehydrogenase mRNA molecules from control and isoproterenol- or dibutyryl cAMP-stimulated glioma cells.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

Partial Purification of Lactate Dehydrogenase mRNA—In our previous publication, we have indicated that lactate dehydrogenase mRNA could be enriched 8-fold by isoproterenol stimulation of confluent C6 glioma cells (11). The stimulation produced a mRNA population in which 0.8% of the sequences coded for the lactate dehydrogenase M-type subunit as estimated by *in vitro* translation. We attempted to enrich the lactate dehydrogenase mRNA by size fractionation of poly(A)⁺RNA on sucrose gradients. Total mRNA activity and lactate dehydrogenase mRNA activity were estimated either after trichloroacetic acid precipitation or after immunoprecipitation of *in vitro* translation products using RNA from selected gradient fractions as templates. Fig. 1 shows the results of a representative sucrose gradient fractionation. Whereas total mRNA activity increases as the size range increases, lactate dehydrogenase mRNA activity displays a sharp peak migrating slightly slower than the 18 S ribosomal RNA peak. Judging by the position of molecular weight markers run on a parallel gradient (rabbit globin mRNA, 18 S and 28 S ribosomal RNA), the size of lactate dehydrogenase mRNA is estimated at 6.0 x 10⁶ daltons. In the peak fractions (fractions 12–14), lactate dehydrogenase accounted for 4% of the total mRNA activity, representing an enrichment of only 5-fold.

Since size distribution of lactate dehydrogenase mRNA relative to total poly(A)⁺RNA was not sufficiently distinct to allow for a meaningful purification by size fractionation, we utilized a modification of the polyribosome immunoprecipitation procedure (44) for the isolation of lactate dehydrogenase mRNA. We observed that it was very important to saturate the polynucleotopes with primary antibody and to separate the polynucleotide/antibody complex from unbound antibody prior to reaction with the GAR-cellulose in order to maximize yield and decrease nonspecific precipitation (results not shown). The enrichment procedure was monitored by using the reticulocyte *in vitro* translation system. Starting with 300 A₂₆₀ units of polyribosomes, we recovered 0.22 mg of polyosomal RNA (Table I). This procedure resulted in an approximately 10-fold enrichment of the lactate dehydrogenase mRNA activity. As shown by translation of this partially purified mRNA in the reticulocyte lysate system and analysis of translated protein by SDS-polyacrylamide gel electrophoresis before and after immunoprecipitation of lactate dehydrogenase (Fig. 2), lactate dehydrogenase mRNA appears to be the predominant mRNA species, although there is a considerable number of contaminating mRNA species.

Synthesis and Molecular Cloning of Double-stranded cDNA—The lactate dehydrogenase-enriched polyosomal RNA was selected for poly(A)⁺RNA by chromatography on oligo(dT)-cellulose prior to oligo(dT)-primed cDNA synthesis. Both yield and size of cDNA were maximized by using saturating levels of reverse transcriptase. Generally, we obtained efficiencies of 15–20% (cDNA synthesized/input RNA) and a modal size of the DNA product of approximately 1500 nucleotides (data not shown). Second strand synthesis by *Escherichia coli* DNA polymerase I utilized the self-primer capability of the first strand (26) and proceeded to 50–75% completion (percentage of first strand). Following S₁ nuclease trimming, the double-stranded cDNA was fractionated on agarose gels after which two size ranges of molecules were recovered. Starting with 2.2 μg of poly(A)⁺RNA, we recovered 4.4 ng of a 1- to 2.5-kbp ds cDNA and 10.6 ng of a 0.5- to 1.0-kbp ds cDNA. The ds cDNA of both size ranges was then de-tailed and annealed with pBR322 that had been D₈-tailed at the *Pst* I site. Transformation of *E. coli* HB101 resulted in 119 Tc⁺ colonies from the 1- to 2.5-kbp material and 331 colonies from the 0.5- to 1.0-kbp cDNA. Since we were interested in generating a cDNA probe of maximal size, only clones with the 1- to 2.5-kbp insert were screened further.

**Screening of Clones for Lactate Dehydrogenase cDNA Sequences**—Replicate plating of Tc⁺ 1- to 2.5-kbp clones en ampicillin-containing agar showed that 104/119 colonies were Tc⁺/Amp⁺. These clones were analyzed by colony hybridization using [³²P]cDNA synthesized from a mRNA population partially enriched for lactate dehydrogenase mRNA by immunoprecipitation of polyribosomes. The mRNA preparation used to generate this screening cDNA probe was, however, isolated from batches of cells different from those used to synthesize the cloned cDNA in an effort to decrease the cross-reactivity with non-lactate dehydrogenase-containing colonies. This preliminary screening identified 11 Tc⁺/Amp⁺ colonies as potentially containing lactate dehydrogenase DNA sequences.
In order to additionally increase the specificity of the filter hybridization analysis, the \(^{32}\text{P}\)cDNA probe used for subsequent colony hybridization was enriched for a predominant cDNA species (45). Since size analysis of the cDNA itself did not yield any distinct species, the cDNA was digested with \textit{Hae} III restriction endonuclease and separated by alkaline agarose gel electrophoresis (46). Autoradiography of the gel identified a distinct band at 500 nucleotides, superimposed on the heterogeneous background of the cDNA (data not shown). The 500-nucleotide cDNA band was recovered from the gel and used in a filter hybridization analysis of the \textit{11 Tc}/\textit{Amp}\(^{+}\) colonies identified by preliminary screening. Using the \textit{Hae} III-restricted \(^{32}\text{P}\)cDNA probe, four clones (Nos. 2, 42, 55, and 97) were identified as containing a predominant cDNA species, presumably lactate dehydrogenase which was the most abundant mRNA present in the RNA preparation used for cDNA synthesis.

The four clones (Nos. 2, 42, 55, and 97) were subsequently analyzed for the size of the inserted DNA fragment. Plasmid DNA was partially purified, digested with \textit{Pst} I, and analyzed on 1.5\% neutral agarose gels. Analysis of the ethidium bromide staining pattern revealed that clones 2, 42, 55, and 97 contained a DNA insert consisting of a 480-bp fragment (see Fig. 3) and a 200-bp fragment (not visible on this gel), thus indicating the presence of an internal \textit{Pst} I site. It appears that the four clones contain plasmids with identical inserts, resulting from a single initial transformation event.

The final screening procedure used for positive identification of lactate dehydrogenase cDNA clones was hybridization-selected translation. Plasmid DNA was purified from clones 2 and 42 and immobilized on DBM paper. After hybridization of poly(A)' RNA from isoproterenol-stimulated C6 glioma cells to the immobilized DNA, the bound RNA was eluted and translated in the reticulocyte lysate cell-free translation system. Translation products were analyzed by SDS-polyacrylamide gel electrophoresis. As shown by the autoradiograph in Fig. 4, RNA eluted from filters containing \textit{pBR322 DNA} (\textit{lane 3}) did not direct the synthesis of any proteins other than the endogenous products of the reticulocyte lysate (\textit{lane 4}). Filters containing plasmid DNA from clones 2 and 42 (\textit{lanes 1 and 2}), however, retained a RNA species which coded for a 35,000-dalton protein (\textit{arrow}) co-migrating with authentic rat liver lactate dehydrogenase (not shown). The identity of this protein was further established by immunoprecipitation with rabbit anti-rat lactate dehydrogenase-\(\beta\) isozyme antiserum. The results (Table II) indicate that clones 2 and 42 hybridized to a mRNA coding for a protein having the antigenic characteristics of rat lactate dehydrogenase M-type subunit. We conclude, therefore, that these recombinant plasmids contain a rat lactate dehydrogenase M-type subunit DNA sequence.

Restriction Endonuclease Analysis of Cloned Lactate Dehydrogenase cDNA—Purified plasmid DNA from clone 42 (\textit{pRLD42}) was further analyzed by digestion with restriction endonucleases as summarized in Fig. 5. The cloned lactate dehydrogenase cDNA appears to be 690 bp in length (assuming 30 bp of \(G\)-C tails at each end). Endonucleases that did not cut the lactate dehydrogenase cDNA include \textit{Eco RI}, \textit{HindIII}, \textit{Bam HI}, \textit{Sam I}, \textit{Sal I}, and \textit{Hha I}.

Northern Blot Analysis of Lactate Dehydrogenase mRNA—Poly(A)’ RNA isolated from either unstimulated C6 glioma cells or cells stimulated for various periods with isoproterenol (10\(^{-5}\) M) was denatured with glyoxal and separated by electrophoresis on a 1.5\% agarose gel. Following transfer of the RNA to nitrocellulose, lactate dehydrogenase mRNA was detected by hybridization to nick-translated \(^{32}\text{P}\)\textit{pRLD42}. The autoradiograph of the Northern blot (Fig. 6) demonstrates that \textit{pRLD42} hybridizes to a RNA species of 5.9 \(\times 10^5\) daltons present in all of the poly(A)' RNA fractions. This value corresponds to approximately 1800 nucleotides, a value which agrees well with our estimate of lactate dehydrogenase mRNA molecular weight obtained by sucrose density gradient centrifugation (Fig. 1). Furthermore, the Northern blot allows a relative quantitation of the amount of lactate dehydrogenase mRNA in the various poly(A)' RNA fractions (49). The autoradiograph (Fig. 6) shows an increased intensity of hybridization of the band corresponding to lactate dehydrogenase mRNA over the time course after isoproterenol-stimulation, although visual inspection appears to indicate that RNA extracted from cells incubated with isoproterenol for 2 h hybridizes more strongly than RNA from cells stimulated for 4 h. However, excision of the bands and quantitation of \(^{32}\text{P}\) radioactivity by liquid scintillation counting (Fig. 7) shows the increase of hybridizable counts in RNA samples from isoproterenol-stimulated C6 glioma cells as a function of stimulation time. The discrepancy between visual inspection of the autoradiograph (Fig. 6) and the actual \(^{32}\text{P}\) counts in the hybrids is not clear but the problem may be due to the geometry of the
FIG. 4. Identification of lactate dehydrogenase recombinant clones by translation of hybrid-selected mRNA. Plasmid DNA (50 μg) was isolated from pBR322 and clones 2 and 42 and immobilized on DBM paper. Poly(A)⁺RNA isolated from C6 glioma cells (stimulated for 8 h with 10⁻⁷ M isoproterenol) was hybridized to the immobilized DNA (15 μg of RNA/filter) for 18 h at 42 °C. The hybridized RNA was eluted and translated in a reticulocyte lysate system. [_¹⁴C]Methionine-labeled protein was subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography. Hybridization-selected RNA translation products are shown in lanes 1 to 3. Lane 1, clone 2 DNA; lane 2, clone 42 DNA; lane 3, pBR322 DNA; lane 4, endogenous products of the reticulocyte lysate system; lane 5, translation products of total glioma cell poly(A)⁺RNA. LDH, lactate dehydrogenase.

FIG. 5. Restriction endonuclease map of the lactate dehydrogenase cDNA insert clone 42 (pRLD42). Numbers indicate the distance in base pairs of the insert from the _Pst_ I site of pBR322 DNA.

hybrid-containing spots on nitrocellulose. The amount of hybridization was maximal in RNA from C6 glioma cells stimulated with 10⁻⁷ M isoproterenol for an 8-h period and declined thereafter. The lactate dehydrogenase mRNA is 2-fold more abundant in poly(A)⁺RNA from 8-h isoproterenol-stimulated glioma cells than in unstimulated cells.

Kinetic Hybridization Analysis—In order to more accurately quantitate the lactate dehydrogenase mRNA levels, we used the cloned cDNA as a probe in a RNA-excess kinetic hybridization analysis of C6 glioma cell poly(A)⁺RNA. The 480-bp fragment of nick-translated, _Pst_ I-digested pRLD42 was isolated and hybridized to poly(A)⁺RNA from either control, isoproterenol-, or dibutyryl cAMP-stimulated C6 glioma cells, under conditions favoring RNA-DNA hybridization (Fig. 8). Comparison of the _R_d values (see Fig. 8) confirms the above Northern blot analysis, showing a 2-fold increase of the number of molecules of lactate dehydrogenase mRNA in poly(A)⁺RNA from either isoproterenol- or dibutyryl cAMP-stimulated C6 glioma cells. Analysis of the computer-fitted _R_d curves also indicates a significant difference in the plateau hybridization values (see Fig. 8). This suggests a partial difference in the nucleotide sequence of the lactate dehydrogenase mRNA present in control versus isoproterenol- or dibutyryl cAMP-stimulated C6 glioma cells.

FIG. 6. Northern blot analysis of C6 glioma cell poly(A)⁺RNA for lactate dehydrogenase mRNA sequences. Confluent C6 glioma cells were stimulated with 10⁻⁷ M isoproterenol for the time periods indicated and poly(A)⁺RNA was isolated. The poly(A)⁺RNA was denatured with glyoxal and subjected to electrophoresis on a neutral 1.5% agarose gel. Following electrophoresis, RNA was transferred to nitrocellulose. Lactate dehydrogenase mRNA was detected by hybridization with nick-translated _³²P_-labeled pRLD42 (clone 42) followed by autoradiography. The numbers shown above each lane refer to the hours of glioma cell stimulation with isoproterenol; C, unstimulated glioma cell RNA. The molecular weight of the LDH mRNA was estimated using HindIII-digested λ DNA and rat ribosomal RNA as standards. The position of 18 S and 28 S RNA is indicated.

FIG. 7. Quantitation of lactate dehydrogenase mRNA after Northern blot analysis. Glioma cell poly(A)⁺RNA was separated by agarose gel electrophoresis and subjected to Northern blot analysis as described in Fig. 6. The bands corresponding to lactate dehydrogenase mRNA were excised from the nitrocellulose sheet, and the hybridized _³²P_ radioactivity was quantitated by liquid scintillation counting. The amount of hybridized radioactivity is displayed as percentage of unstimulated control.
Although CAMP or effector agents that generate CAMP are known to induce a number of proteins in eukaryotes (1, 2, 4–11), the molecular mechanisms of induction are virtually undisclosed. Recently, our laboratory has investigated the catecholamine-mediated induction of lactate dehydrogenase in the rat C6 glioma cell line as a model system to elucidate the role of CAMP in eukaryotic enzyme induction. In the present study, we have generated a specific cDNA probe complementary to lactate dehydrogenase mRNA through the technique of molecular cloning and have utilized this probe to quantitate lactate dehydrogenase mRNA levels in rat C6 glioma cells stimulated with either isoproterenol or dibutyryl cAMP.

Our strategy consisted of purification of lactate dehydrogenase mRNA, generation of cDNA, and amplification of this cDNA through molecular cloning. Partial purification of lactate dehydrogenase mRNA was achieved through immunoprecipitation of polyribosomes isolated from isoproterenol-stimulated C6 glioma cells, resulting in a mRNA preparation in which lactate dehydrogenase mRNA represented 20% of the total mRNA activity as evaluated by in vitro translation analysis. The relative efficiency of this purification procedure is demonstrated by the facts that it resulted in a 10-fold enrichment of lactate dehydrogenase mRNA as compared to mRNA which was not isolated by polyisomunoprecipitation and in a 200-fold enrichment as compared to the lactate dehydrogenase mRNA activity in poly(A)+RNA preparations from unstimulated glioma cells.

The technique of molecular cloning was utilized to amplify and purify cDNA prepared from this partially purified lactate dehydrogenase mRNA. A crude [3H]cDNA probe generated from the partially enriched lactate dehydrogenase mRNA was used for the initial screening of recombinant clones by filter hybridization. This initial screening identified a subset of bacterial colonies which potentially contained lactate dehydrogenase cDNA sequences. Further screening of these colonies was accomplished after enrichment of the cDNA probe for an abundant sequence (45) prepared by Hae III digestion of the cDNA probe and isolation of a 500-nucleotide fragment. Use of this restriction fragment as a hybridization probe allowed us to identify 4 bacterial colonies as putative lactate dehydrogenase cDNA clones. Size fractionation of Pet 1 digests of plasmid DNA isolated from the 4 colonies identified the latter as containing identical plasmids. The presence of lactate dehydrogenase DNA sequences in these plasmids was confirmed by hybridization-selected in vitro translation resulting in the synthesis of a protein which was identified as lactate dehydrogenase on the basis of its molecular size and its specific reaction with rabbit anti-rat lactate dehydrogenase-5 isozyme antiserum. Restriction endonuclease analysis indicated the cloned lactate dehydrogenase cDNA to be approximately 700 nucleotides in length.

The use of the cloned cDNA probe in a Northern blot analysis of electrophoretically separated denatured poly(A)+RNA indicated lactate dehydrogenase mRNA to be about 1800 nucleotides in length. Excluding a poly(A) sequence of about 150 nucleotides, the composite of the translated and untranslated regions of lactate dehydrogenase mRNA is thus formed by about 1650 nucleotides. Based on $M_r = 35,000$ for the in vitro translation product of lactate dehydrogenase mRNA, approximately 600 bases form the untranslated region of the mRNA. The relative size of this untranslated region is similar to that observed for a number of other proteins (50–54).

Using the discriminating ability of the cDNA probe to select specifically for lactate dehydrogenase mRNA, we have demonstrated by Northern blot as well as by kinetic hybridization analysis that isoproterenol (or dibutyryl CAMP) stimulation of C6 glioma cells causes a 2-fold increase in the number of lactate dehydrogenase mRNA molecules 8 h after addition of the inducing agents. Although the rate of accumulation of lactate dehydrogenase mRNA after isoproterenol stimulation followed a time course similar to the one previously determined by us using in vitro translation of poly(A)+RNA isolated from isoproterenol-stimulated glioma cells (exhibiting maximal activity 8 h after stimulation) (11), we previously found an 8- to 10-fold maximal increase of the functional level of lactate dehydrogenase. Since we have ruled out technical problems in either the hybridization or translation assays (all analyses were repeatedly carried out under different conditions using the same poly(A)+RNA preparations), this discrepancy in our finding suggests that isoproterenol or dibutyryl CAMP not only cause an increase of the number of lactate dehydrogenase mRNA molecules but that the newly induced mRNA exhibits a higher translational efficiency per molecule of mRNA than lactate dehydrogenase mRNA from unstimulated glioma cells. The increased translation efficiency could be the result of factors present in the induced poly(A)+RNA fraction allowing for more effective utilization of lactate dehydrogenase mRNA or could be the result of inherent structural modifications of the mRNA.

The reproducible significant difference observed in the plateau hybridization values after kinetic hybridization of poly(A)+RNA (Fig. 8) suggests that lactate dehydrogenase mRNA from isoproterenol- or dibutyryl cAMP-stimulated cells exhibits a higher degree of homology with the cloned DNA template (which was prepared from mRNA of stimulated cells) than mRNA from unstimulated cells. Since the products of in vitro translation of lactate dehydrogenase mRNA from unstimulated and stimulated cells are identical (11), the difference in homology may reflect significant base sequence changes in the untranslated region of lactate dehydrogenase mRNA from stimulated cells as compared to mRNA from unstimulated cells. While our data do not prove a transcriptional role for cAMP, the model we consider pre-
dicts an action of cAMP upon mRNA transcription and/or post-transcriptional processing with subsequent translational effects resulting from a modified mRNA, that might show an increased rate of polypeptide initiation and/or elongation. Obviously, cAMP may be acting at different molecular levels depending on the particular biological model system, but the accumulation of a more translationally efficient lactate dehydrogenase mRNA may help to explain some of the discrepancies observed after cAMP induction of tyrosine aminotransferase in rat liver (1, 14-17).

REFERENCES

1. Wicks W. D. (1974) Adv. Cyclic Nucleotide Res. 4, 335-438
2. Rosenfeld, M. G., and Barrieux, A. (1979) Adv. Cyclic Nucleotide Res. 11, 206-294
3. Pastan, I., and Adhya, S. (1976) Bacteriol. Res. 40, 507-515
4. Lamartiniere, C. A., and Feigelson, M. (1977) J. Biol. Chem. 252, 3234-3239
5. Steinberg, R. A., and Coffino, P. (1979) Cell 18, 713-733
6. Suleiman, S., and Vestling, A. Cyclic Nucleotide Res. 4, 1712
7. Prashad, N., Rosenberg, R. N., Wischmeyer, B., Ulrich, C., and Steinberg, R. A., and Coffino, P. (1978) J. Biol. Chem. 253, 319-322
8. Prasad, N., Rosenberg, R. N., Wischmeyer, B., Ulrich, C., and Sparkman, D. (1979) Biochemistry 18, 2717-2725
9. Kumar, S., McGinnis, J. F., and de Vellis, J. (1980) J. Biol. Chem. 255, 2515-2521
10. Brown, P. C., and Papaconstantinou, J. (1979) J. Biol. Chem. 254, 9379-9384
11. Bollino, H. C., and Dolly, J. (1979) Nucleic Acids Res. 6, 5205-5209
12. Ramsey, J. C., and Steele, W. J. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 1020-1025
13. Palmiter, R. D., Palacios, R., and Schimke, R. T. (1977) J. Biol. Chem. 524, 1020-1025
14. O’Brien, R. C., and Papaconstantinou, J. (1979) J. Biol. Chem. 254, 5374-5384
15. van de Pol, K. W., van Aken, J. M., and van Wijk, R. (1979) Cell Biol. Int. Rep. 3, 347-355
16. Jungmann, R. A., Christensen, M. L., and Derda, D. F. (1979) in Effects of Drugs on the Cell Nucleus (Busch, H., Crooke, S. T., and Daskal, V. eds) pp. 507-519, Academic Press, New York
17. Harrison, J. J., Suter, P., Suter, S., and Jungmann, R. A. (1980) Biochem. Biophys. Res. Commun. 94, 1265-1269
18. Palmiter, R. D., Oka, T., and Schimke, R. T. (1971) J. Biol. Chem. 246, 724-737
19. Gough, N. M., and Adams, J. M. (1978) Biochemistry 17, 5560-5566
20. Ramsey, J. C., and Steele, W. J. (1976) Biochemistry 15, 1704-1712
21. Schutz, G., Kieval, S., Croner, B., Sippel, A. E., Kurtz, D. T., and Feigelson, P. (1977) Nucleic Acids Res. 4, 71-84
22. Stein, J. P., Catterall, J. F., Woo, S. L. C., Means, A. R., and O’Malley, B. W. (1978) Biochemistry 17, 5763-5772
23. Kacian, D. L., and Meyera, J. C. (1976) Proc. Natl. Acad. Sci. U. S. A. 74, 2191-2195
24. Efstratiadis, A., Kafatos, F. C., Maxam, A. M., and Maniatis, T. (1976) Cell 7, 279-288
25. Langridge, J., Langridge, P., and Berquist, P. L. (1980) Anal. Biochem. 103, 264-271
26. Boliver, R., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heynecker, H. L., and Boyer, H. W. (1977) Gene 2, 95-113
27. Roychoudhury, R., Jay, E., and Wu, R. (1976) Nucleic Acids Res. 3, 863-877
28. Dagnet, M., and Ehrlich, S. D. (1979) Gene 6, 23-28
29. Thayer, H. E. (1979) Anal. Biochem. 86, 60-63
30. Denhardt, D. T. (1966) Biochem. Biophys. Res. Commun. 29, 641-646
31. Birnboim, H. C., and Doly, J. (1979) Nucleic Acids Res. 7, 1513-1523
32. Guerry, P., LeBlanc, D. J., and Falkow, S. (1973) J. Bacteriol. 116, 1064-1066
33. Clewell, D. B., and Helsinski, D. R. (1969) Proc. Natl. Acad. Sci. U. S. A. 62, 1159-1166
34. Smith, H. O., and Birnstiel, M. L. (1976) Nucleic Acids Res. 3, 2387-2398
35. Alwine, J. C., Kemp, D. J., and Stark, G. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 72, 3961-3975
36. McMaster, G. K., and Carmichael, G. G. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 4835-4838
37. Thomas, P. S. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 5201-5205
38. Higby, P. W. J., Dieckmann, M., Rhodes, R., and Berg, P. (1977) J. Mol. Biol. 113, 237-251
39. Casev, J., and Davidson, N. (1977) Nucleic Acids Res. 4, 1539-1552
40. Duggleby, R. G. (1981) Anal. Biochem. 110, 9-18
41. Galau, G. A., Britten, R. J., and Davidson, E. H. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 1020-1025
42. Palmiter, R. D., Palacios, R., and Schimke, R. T. (1972) J. Biol. Chem. 247, 3296-3304
43. Seeburg, P. H., Shine, J., Martial, J. A., Ulrich, A., Baxter, J. D., and Goodman, H. M. (1977) Cell 12, 157-171
44. McDonell, M. W., Simon, M. N., and Studier, F. W. (1977) J. Mol. Biol. 110, 119-146
45. Stark, G. R., and Williams, J. G. (1979) Nucleic Acids Res. 6, 195-203
46. Smith, D. F., Searle, P. F., and Williams, J. G. (1979) Nucleic Acids Res. 6, 487-506
47. Adams, S. L., Alwine, J. C., de Crombrugghe, B., and Pastan, I. (1979) J. Biol. Chem. 254, 4935-4938
48. McReynolds, L., O’Malley, B. W., Nieb, A. D., Fothergill, J. E., Givol, D., Fields, S., Robertson, M., and Brownlee, G. G. (1978) Nature 273, 723-728
49. Gordon, J. I., Burns, A. T. H., Christmann, J. L., and Deyel, R. G. (1975) J. Biol. Chem. 253, 8629-8639
50. Schwartz, R. J., Haron, J. A., Rothblum, K. N., and Dugiczyk, A. (1980) Biochemistry 19, 5883-5900
51. Benyajati, C., Wang, N., Reddy, A., Weinberg, E., and Sofer, W. (1989) Nucleic Acids Res. 8, 5649-5667
52. Groner, B., Hynes, N. E., Sippel, A. E., Sjö, S., Hurr, M. C. N., and Schütz, G. (1977) J. Biol. Chem. 252, 6666-6674
53. Payvar, S., and Schinke, R. T. (1979) Eur. J. Biochem. 101, 271-282
54. McKnight, G. S., and Palmiter, R. D. (1979) J. Biol. Chem. 254, 9050-9058
Cyclic AMP and Lactate Dehydrogenase mRNA

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EXPERIMENTAL PROCEDURES

Preparation of Affinity-Purified Antibodies - Purified rat liver lactate dehydrogenase-5 monoclonal antibody was directed against 55 kDa purified rabbit IgG from the hybridoma culture. The antibody was affinity purified using 125 kDa lactate dehydrogenase-5 coupled to CNBr-Sepharose (Pharmacia Fine Chemicals) as described by the manufacturer. The antibody was used at a 1:1 ratio in a 1:1 mixture of 2 volumes antibody and 1 volume of 10X wash buffer (0.1 M Tris, 0.1 M NaCl, 0.05% Tween 20, 0.05% NaN3, 0.005% NP-40)

Isolation of Polyribosomes - Polyribosomes were isolated by a modification of the procedure of Darnell, Sambrook and Maniatis. Polyribosomes were prepared from the liver cytosol corresponding to 1 g of fresh liver. The cytosol was incubated with a 300 fold excess of recombinant lambda deoxyribonuclease (150 U/mg) for 10 min at 37°C. The resulting 500 fold excess of recombinant lambda deoxyribonuclease (150 U/mg) for 10 min at 37°C. The resulting nucleic acid fraction was then sonicated to release the polyribosomes.

Immunodetection of Lactate Dehydrogenase mRNA-Containing Polyribosomes - Polyribosomes containing lactate dehydrogenase mRNA were isolated by the immunoprecipitation procedure of Darnell, Sambrook and Maniatis. The polyribosomes were sonicated and then digested with pancreatic RNase A and T1. The resulting mixture was then treated with RNA polymerase and transferred to a 37°C incubation mixture. The resulting mixture was then treated with RNA polymerase and transferred to a 37°C incubation mixture.

Preparation of Recombinant Plasmids and Transformation of E. coli - Recombinant plasmids were prepared by the method of Sambrook, Maniatis and Darnell. The plasmids were then transformed into E. coli by the method of Darnell, Sambrook and Maniatis.

Preparation of Affinity-Purified Antibodies - Purified rabbit IgG was directed against 55 kDa purified rabbit IgG from the hybridoma culture. The antibody was affinity purified using 125 kDa lactate dehydrogenase-5 coupled to CNBr-Sepharose (Pharmacia Fine Chemicals) as described by the manufacturer. The antibody was used at a 1:1 ratio in a 1:1 mixture of 2 volumes antibody and 1 volume of 10X wash buffer (0.1 M Tris, 0.1 M NaCl, 0.05% Tween 20, 0.05% NaN3, 0.005% NP-40).

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Cyclic AMP and Lactate Dehydrogenase mRNA

TABLE I

| Fraction | Total LDH mRNA activity | LDH mRNAs | LDH mRNA activity |
|----------|-------------------------|-----------|------------------|
| RNA      | % of total mRNA activity | activity |
| mg 10^7 pm |                      | ng | pm | ng |
| Total     | 10.6 | 6.1 | 1.9 | 4352 |
| Immunoprecipitated RNA | 0.70 | 0.7 | 21.0 | 68602 |

1. RNA was translated in the rabbit reticulocyte lysate system and lactate dehydrogenase was detected after immunoprecipitation and SDS-polyacrylamide gel electrophoresis of the immunoprecipitate as described previously (11).
2. Total mRNA activity was estimated after trichloroacetic acid precipitation of the translation products and determination of 32P radioactivity (11).
3. Poly(A) RNA was prepared from confluent C6 glioma cell cultures which had been stimulated with hydrocortisone (10^-6 M) for 24 h.
4. Total C6 glioma RNA hybridized with lactate dehydrogenase mRNA by the double isotope immunoradiometric technique as described under "Experimental Procedures".

TABLE II

| RNA | % of C6 immunoprecipitated lactate dehydrogenase |
|-----|-----------------------------------------------|
| Endogenous reticulocyte lysate RNA | 0.0 |
| Total C6 glioma poly(A)^+RNA | 0.6 |
| Hybrid-selected RNA from pBR322 | 3.0 |
| Hybrid-selected RNA from clone 22 | 3.8 |
| Hybrid-selected RNA from clone #2 | 7.3 |

Poly(A)^+RNA and hybrid-selected mRNAs were obtained and translated in a reticulocyte lysate system as described in the legend to Figure 4. Hybrid-selected labeled lactate dehydrogenase was immunoprecipitated as described (11) and subjected to SDS-polyacrylamide gel electrophoresis. Reactivity in gel slices corresponding to lactate dehydrogenase was determined as described under "Experimental Procedures".

Figure 1:

Quantitation of poly(A)^+RNA isolated from rat C6 glioma cells stimulated with isoproterenol (10^-5 M) for 8 h. Poly(A)^+RNA was prepared and centrifuged through a 15 to 30% sucrose gradient. C6 glioma mRNAs and total mRNA activity were determined by the precipitation of total [32P]-labeled poly(A)^+ labeled gradient. The mRNAs were eluted from the gradient and the positions of the poly(A)^+ RNA markers were 0.0, 0.10.

| Fraction | Total mRNA Activity |
|----------|---------------------|
| RNA      | % of total activity |
| mg 10^7 pm |                      | ng | pm | ng |
| Total     | 10.6 | 6.1 | 1.9 | 4352 |
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