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MOLECULAR CLONING OF DNA COMPLEMENTARY TO mRNA OF RAT LIVER SERINE DEHYDRATASE*

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SUMMARY: A cDNA clone containing sequences complementary to the mRNA cording for rat hepatic serine dehydratase was isolated to study the multihormonal regulation of this enzyme. Serine dehydratase mRNA was partially purified (50-fold enrichment, 8.2 % of the total mRNA activity) from the liver of rats fed high protein diet by polysome immunoadsorption followed by oligo(dT)-cellulose column chromatography. This preparation was used as template for synthesis of cDNA. Double-stranded cDNA sequences were inserted into the plasmid pBR322 and cloned in Escherichia coli DH1. Of 860 transformants screened, 6 clones containing DNA complementary to serine dehydratase mRNA were identified by differential colony hybridization and hybrid-selected translation. The length of serine dehydratase mRNA was estimated to be 1,500 bases by Northern blot analysis. One cloned cDNA comprised about 1,000 base pairs, or 65 % of the length of the mRNA. The amount of the mRNA was greatly increased in the liver of rats given high protein diet.

Serine dehydratase (EC 4.2.1.13, SDH) is located specifically in the liver and is induced in the gluconeogenic state. In primary cultures of adult rat hepatocytes, we found that SDH mRNA activity, assayed in a cell-free translation system, was regulated by various hormones; namely, dexamethasone plus glucagon increased the level of translatable SDH mRNA about 10 times, whereas insulin or catecholamines via α_1-adrenegic receptor suppressed this increase (1). A unique feature of the induction of this enzyme is that, unlike in the cases of tryptophan oxygenase (2,3) and tyrosine aminotransferase (4), neither dexamethasone nor glucagon alone increases SDH mRNA activity: that is the induction of SDH requires both dexamethasone and glucagon(1,5-7). Therefore, it seemed interesting to study the mechanism of regulation of gene expression

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Abbreviations: SDH, serine dehydratase; cAMP, cyclic AMP.

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of SDH in comparison with those of tryptophan oxygenase and tyrosine aminotransferase. For this purpose, it was necessary to obtain a specific cDNA probe for use in hybridization assay.

Here we describe the construction of a SDH cDNA clone and its use in analysis of the size of SDH mRNA from rat liver. Our results indicate that the level of SDH mRNA is increased in the livers of rats given a high protein diet.

MATERIALS AND METHODS

Materials --- The following materials were used: AMV reverse transcriptase (Bio-Rad), the Klenow fragment of DNA polymerase I, S1 nuclease, oligo(dT)12-18, terminal deoxynucleotidyl transferase and oligo(dG)-tailed pBR322 (Pharmacia P-L Biochemicals), protein A-Sepharose Cl-4B (Pharmacia), oligo(dT)-cellulose (Collaborative Research), deoxyribonucleotides (Takara Shuzo Co.), [35S]methionine (1,100 Ci/mmol), a reticulocyte lysate translation kit (Radiochemical Centre, Amersham) and Staphylococcus aureus (Calbiochem-Behring). [α-^{32}P]dCTPs (600 Ci/mmol and 3,000 Ci/mmol) for synthesis of cDNA and nick translation, respectively, and ENLIGHTNING were purchased from New England Nuclear.

Purification of SDH mRNA by Immunoadsorption --- Polysomes from the livers of rats that had received a 75 % high protein diet for 7 days, were isolated by sedimentation through a discontinuous sucrose gradient by the procedure of Kraus and Rosenberg (8). One g of liver yielded 30-50 A260 units of polysomes. The polysome preparation was incubated for 2 h at 4°C with pufified, monospecific IgG against SDH (1 mg/50 A260). Antibody-nacent chain-polysome complexes were adsorbed to a protein A-Sepharose column and after extensive washing, the bound RNA was eluted with a buffer containing 25 mM Tris, 20 mM EDTA, pH 7.5, and heparin (0.2 mg/ml). The poly(A$^+$) RNA was then separated from polysomal RNA on an oligo(dT)-cellulose column (8). For estimation of mRNA activity, RNA extracted from the polysome preparation by the SDS-phenol procedure (9,10) or purified poly(A$^+$) RNA was translated in a reticulocyte lysate system (11).

Synthesis of Double-stranded cDNA and Construction of cDNA Plasmids --- Immunoenriched mRNA (2.5 µg) was used as a template for synthesis of cDNA by standard methods (12). After S1 nuclease treatment, blunt duplex cDNA was tailed with 20 dC residues using terminal transferase and annealed to PstI-cleaved oligo(dG)-tailed pBR322. Escherichia coli DH1 cells were transformed by a reported method (13). Colonies containing recombinant plasmids were identified by their resistance to tetracycline and sensitivity to ampicillin.

Screening of the cDNA Library by Differential Colony Hybridization --- Transformants from the cDNA library were grown and fixed on nitrocellulose filters for in situ colony hybridization (14). Differential screening was performed by hybridizing duplicating filters with single stranded [^{32}P]-labeled cDNA (approximately 9 x 10^5 cpm/ml) synthesized from polysomal poly(A$^+$) RNA from the livers of rats fed on either a normal or high protein diet. Colonies that gave a differential signal were screened further by hybridization with enriched [^{32}P]-labeled cDNA, which was prepared from immunopurified poly(A$^+$) RNA.

Hybrid-Selected Translation --- Plasmid DNA was isolated by a SDS-alkali procedure, essentially as described (15), and contaminating RNA was removed by precipitating the plasmid DNA with polyethylene glycol (16). Hybrid-selection assays were carried out as described (17). Samples of 5 µg of recombinant plasmid DNA or pBR322, which were not linearized, were spotted onto nitrocellulose filters. The filters were incubated for 3 h at 50°C with 25 µg of poly(A$^+$) RNA isolated from the liver of rats fed on high protein

diet and rinsed before elution of the hybridized mRNA. The hybrid selected mRNA was translated in a reticulocyte lysate system (11).

Electrophoresis and Northern Blot Hybridization of mRNA --- Poly(A^+) RNA was treated with glyoxal, subjected to electrophoresis in 1.1 % agarose gel, transferred to a nitrocellulose filter, and hybridized with nick-translated, [^{32}P]-labeled cDNA probe as described (18).

Nick Translation --- The plasmid DNA, pSDH4, was digested with PstI enzyme and purified on low-melting-temperature agarose. The larger (ca. 780 base pairs) of the fragments of this insert was nick-translated with [^{32}P]-dCTP (3,000 Ci/mmol) to 3 x 10^7 cpm/μg essentially as described (19).

RESULTS

Purification of mRNA for SDH by Polysome Immunoadsorption --- SDH mRNA has been estimated by an in vitro translational assay to represent only 0.008 % or less of the poly(A^+) RNA, extracted with SDS-phenol from normal rat liver, but its content increased to 0.03 to 0.05 % when rats were given a high protein diet. About 3-fold enrichment of this mRNA was achieved when RNA was extracted from the polysomal fraction. Because of the low concentration of SDH mRNA even in induced conditions, we employed an immunoadsorption procedure followed by oligo(dT)-cellulose column chromatography for its isolation and obtained 10 μg of enriched poly(A^+) RNA from 10 g of liver. The purity of this SDH mRNA was estimated to be 8.2 % by *in vitro* translation assay, representing approximately 50-fold enrichment from the starting polysome preparation.

Construction and Identification of cDNA Clones for SDH --- Double-stranded cDNA was synthesized from SDH mRNA-enriched poly(A^+) RNA and inserted into the PstI site of pBR322 by oligo(dC)-oligo(dG) tailing. The resultant recombinant plasmids were used to transform *Escherichia coli* DH1. From 2.5 μg of enriched poly(A^+) RNA, 0.27 μg of (dC)-tailed double stranded cDNA was obtained, and approximately 1,000 tetracycline-resistant and ampicillin-sensitive colonies were produced by one ng of the (dC)-tailed double-stranded cDNA. DNA from colonies fixed on nitrocellulose filters representing 860 transformants were initially screened for sequences complementary to SDH mRNA by differential colony hybridization. Duplicate filters were probed with [^{32}P]-labeled cDNA synthesized from hepatic mRNA of normal rats or induced mRNA of from rats given a high protein diet. One hundred differential signals were identified

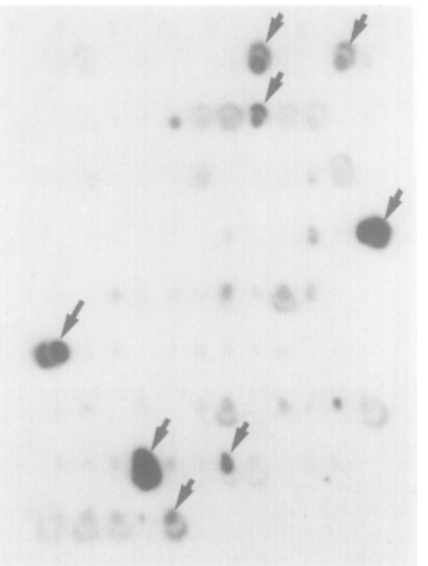

Fig. 1. Screening of clone containing sequences homologous to SDH mRNA by colony hybridization. The nitrocellulose filter contained 100 colonies, which were initially screened by differential colony hybridization. The filters were hybridized with [^{32}P]cDNA synthesized from immunopurified SDH mRNA from rat liver. Arrows indicate clones subsequently subjected to hybrid-selected translation.

by screening the 860 colonies. These colonies were further screened by hybridization with [^{32}P]-labeled cDNA synthesized from the enriched mRNA. Three signals of high intensity and five signals of moderate intensity were observed with the enriched probe, as shown in Fig. 1.

For confirmation that these colonies actually contained a cDNA sequences homologous to that of SDH mRNA, plasmid DNA was isolated from the eight transformants and fixed to nitrocellulose for hybrid-selected translation. As shown in Fig. 2, plasmid DNA prepared from six colonies selected for mRNA encoded a translational product with an apparent molecular weight of 34,000 (lanes 1 to 6) which correspond to the molecular weight of SDH synthesized in this *in vitro* system from poly(A$^+$) RNA isolated from the liver of rat given a high protein diet (lane 9) and also to the molecular weight of purified SDH subunit (20), while plasmid DNA from the other two colonies did not (lanes 7 and 8). Substitution of the plasmid DNA with pBR322 did not produce immunoprecipitable SDH (lane 10). These results indicated that the six colonies contained specif-

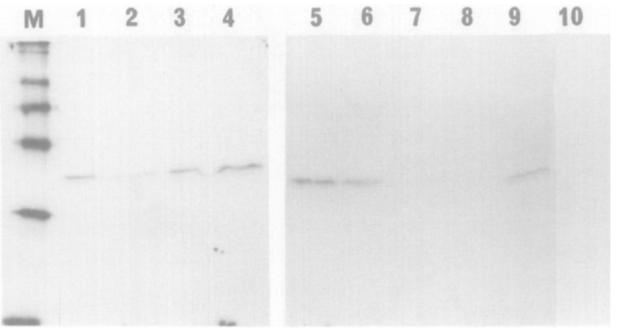

Fig. 2. Identification of SDH cDNA by hybrid-selected translation. Recombinant plasmid DNAs (lanes 1 to 8) or pBR322 (lane 10) were separatedly bound to nitrocellulose filters and hybridized to poly(A^+) RNA extracted from the livers of rats given high protein diet. The hybridized mRNA was eluted and translated in 16 µl of reticulocyte lysate system. The [^{35}S]labeled translated products were immunoprecipitated with anti-SDH antibody and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Lane 9 shows the protein that was translated directly from poly(A^+) RNA and immunoprecipitated with anti-SDH antibody. Lane M shows the position of the [^{14}C]methylated molecular weight standards, myosin(200,000), phosphorylase b(92,500), bovine serum albumin (69,000), ovalbumin(46,000), carbonic anhydrase(30,000) and lysozyme(14,300).

ic sequences of DNA that are complementary to mRNA of SDH. These colonies were named pSDH1 to pSDH4.

Size of cDNA Inserts --- The plasmid DNAs containing SDH cDNA were digested with PstI restriction enzyme and analyzed on 1.5 % agarose gel. All the inserts except that of pSDH5 yielded two bands on agarose gel (Fig. 3, lanes 1,2,3,4, and 6), indicating that SDH cDNA sequences have at least one PstI restriction site. The faint bands just below the short inserts must be those of contaminating RNA. Of the six clones, pSDH4 contained the largest insert of 780 plus 250 base pairs (total about 1,000 base pairs).

Estimation of the Size of SDH mRNA --- Samples of poly(A^+) RNA from the livers of rats given normal and high protein diets were denatured with glyoxal and subjected to electrophoresis on 1.5 % agorose gel to separate the RNA species according to size. The resolved RNA was transferred to nitrocellulose paper and hybridized with the [^{32}P]-labeld cDNA insert excised from pSDH4. With this probe a polynucleotide of approximate 1,500 bases was identified as SDH mRNA extracted from the livers of rats given high protein diet (Fig. 4, lane 2), but this polynucleotide was scarcely detectable in mRNA from the

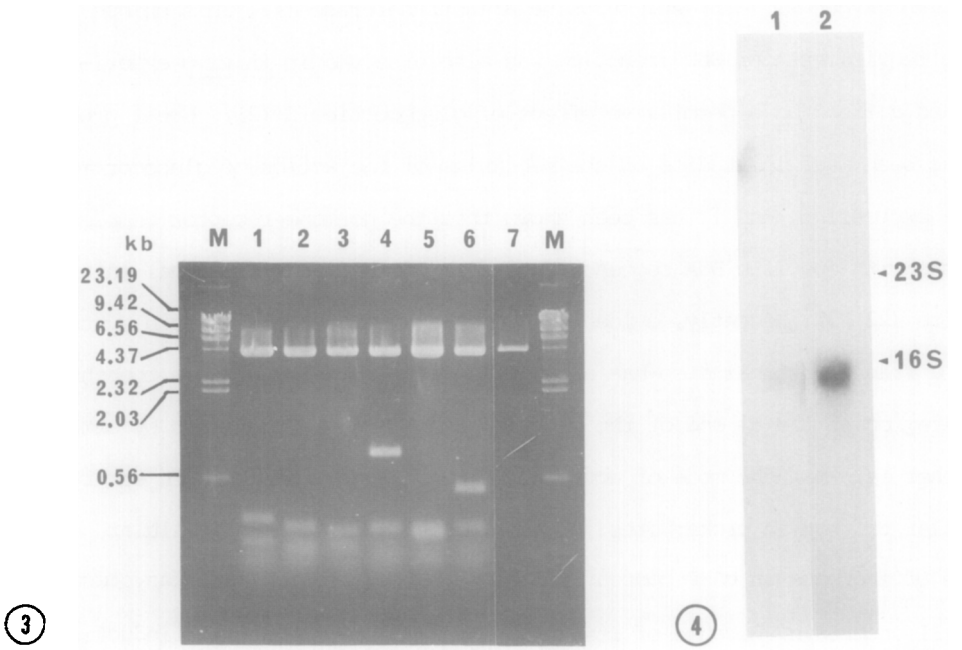

Fig. 3. Size of cloned SDH cDNAs. Plasmid DNAs extracted from 6 clones (lanes 1 to 6) or pBR322 (lane 7) were digested with PstI and subjected to electrophoresis on 1.5 % agarose gel. The DNAs were stained with ethidium bromide and photographed under ultraviolet light.

Fig. 4. Determination of SDH mRNA size. Poly(A^+) RNA samples were subjected to electrophoresis under denatured conditions as described under "MATERIALS AND METHODS". The RNA was blot-transferred to nitrocellulose and hybridized with [^{32}P]labeled pSDH4.

livers of rats given normal diet (lane 1). However, the mRNA from uninduced rat liver could be clearly detected on an autoradiogram by prolongation of the exposure time, and its size was the same as that of the mRNA from induced rat liver (data not shown). These findings indicate that high protein diet, probably by increasing the secretions of glucocorticoids and glucagon, caused marked increase in the absolute amount of SDH mRNA. Assuming that a coding sequence of about 960 nucleotides is required for a protein of the SDH subunit, which has a molecular weight of 34,000 (20), the other nucleotides (about 540 nucleotides) could be those of 3' and 5' non-coding regions and a poly-adenylated moiety of the mRNA.

DISCUSSION

We showed previously that induction of SDH mRNA requires both dexamethasone and glucagon (1,5-7), and that this hormone requirement differs from those of

tryptophan oxygenase (2,3) and tyrosine aminotransferase (4). This unique hormone requirement for SDH induction was also observed in in vivo experiments and defined as a permissive effect of glucocorticoid (21). Great progress has been made in studies on the mechanism of the action of glucocorticoid on gene expression, and it has been shown that the hormone-receptor complex interacts with specific DNA sequences upstream of the transcriptional initiation site (22,23). Recently, evidence that cAMP also controls protein synthesis at a transcriptional step has accumulated (24-28) and it is now thought that a region at the 5' end of the gene is essential for regulation by cAMP (29); that is, that the mode of action of cAMP on gene expression in eukaryotes is similar to that in prokaryotes. However, details of the intracellular effects of increase in cAMP concentration on gene expression in eukaryotes are still uncertain. Because of its unique hormone requirement for induction, SDH is a good marker enzyme to use in studies on the molecular mechanism of the cooperative effect of glucocorticoid and glucagon on gene expression.

It is difficult to study the mechanism of action of these hormones in vivo, owing to their complex indirect effects, but we showed that primary cultures of hepatocytes are a suitable system for such studies (1-7). In this work, we obtained cloned SDH cDNA from rat liver. By hybridization with this cloned cDNA as a probe, we can now measure directly the amounts of SDH mRNA in cultured hepatocytes treated with various hormones and the rate of transcription of the SDH gene in isolated nuclei.

REFERENCES

1. Noda,C., Tomomura,M., Nakamura,T. & Ichihara,A. (1984) J. Biochem. 95, 37-45.
2. Nakamura,T., Shinno,H. & Ichihara,A. (1980) J. Biol. Chem. 255, 7533-7535.
3. Niimi,S., Nakamura,T., Nawa,K. & Ichihara,A. (1983) J. Biochem. 94,1697-1706.
4. Nakamura,T., Noda,C. & Ichihara,A. (1981) Biochem. Biophys. Res. Commun. 99, 775-780.
5. Noda,C., Nakamura,T. & Ichihara,A. (1983) J. Biol. Chem. 258, 1520-1525.
6. Noda,C., Shinjyo,F., Nakamura,T. & Ichihara,A. (1983) J. Biochem. 93, 1677-1684.
7. Noda,C., Nakamura,T. & Ichihara,A. (1981) Biochem. Biophys. Res. Commun. 100, 65-72.
8. Kraus,J.P. & Rosenberg,L.E. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 4015-4019.

9. Bynum,J.W. & Ronzio,R.A. (1976) Anal. Biochem. 73,209-214.
10. Mori,M., Miura,S., Tatibana,M. & Cohen,P.P. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 5071-5075.
11. Pelham,H.R.B. & Jackson,R.T. (1976) Eur. J. Biochem.67, 247-256.
12. Maniatis,T., Fritsch,E.F. & Sambrook,J. (1982) Molecular Cloning: A Laboratory Manual, pp. 229-242, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
13. Hanahan,D. (1983) J. Mol. Biol. 166, 557-580.
14. Maniatis,T., Fritsch,E.F. & Sambrook,J. (1982) Molecular Cloning: A Laboratory Manual, p.314, p.326,Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
15. Birnboim,H.C. & Doly,J. (1979) Nucleic Acid Res. 7, 1513-1523.
16. Lis,J.T. & Schleif,R. (1975) Nucleic Acid Res. 2. 383-389.
17. Maniatis,T., Fritsch,E.F. & Sambrook,J. (1982) Molecular Cloning: A Laboratory Manual, pp. 329-333, Cold Spring Harbor Laboratory, Cold Spring Harbor,New York.
18. Thomas,P.S. (1983) Methods Enzymol. 100, 255-267.
19. Rigby,P.W.J., Dieckmann,M., Rhodes,C. & Berg,P. (1977) J. Mol. Biol. 113, 237-251.
20. Inoue,H., Kasper,C.B. & Pitot,H.C. (1971) J. Biol. Chem. 246, 2626-2632.
21. Ishikawa,E. & Nakajima,K. (1970) J. Biochem. 67, 685-692.
22. Yamamoto,K.R. & Alberts,B.M. (1976) Ann. Rev. Biochem. 45, 721-746.
23. Anderson,J.N. (1984) The effect of steroid hormones on gene transcription. in Biological Regulation and Development, Vol. 3B, Hormone Action (Goldberger,R.F. & Yamamoto,K.R., eds) pp. 169-212, Plenum Press, New York.
24. Meisner,H.,Lamers,W.H. & Hanson,R,W. (1983) Trends Biochem. Sci. 8, 165-167.
25. Maurer,R.A. (1981) Nature 294, 94-97.
26. Murdoch,G.H., Rosenfeld,M.G. & Evans,R.M. (1982) Science 218, 1315-1317.
27. Jungmann,R.A., Kelley,D.C., Miles,M.F. & Milkowski,D.M. (1983) J. Biol. Chem. 258, 5312-5318.
28. Hashimoto,S., Schmid,W. & Schütz,G. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 6637-6641.
29. Wynshaw-Boris,A., Lugo,T.G., Short,J.M., Fournier,R.E.K. & Hanson,R.W. (1984) J. Biol. Chem. 259, 12161-12169.