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INCREASED MESSENGER RNA CONCENTRATION FOR CARBAMOYL-PHOSPHATE SYNTHASE II IN HEPATOMA 3924A*

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Previous investigations demonstrated that carbamoyl-phosphate synthase II (synthase II) (EC 6.3.5.5) activity, amount, and in vivo synthetic rate increased approximately 9-fold in the rapidly proliferating rat hepatoma 3924A compared to normal liver (1-4). This study provides evidence by Northern and RNA dot blot hybridizations of a 13-fold increase in the amount of hepatoma 3924A synthase II mRNA compared to levels in normal liver. Southern and DNA dot blots indicated amplification of CAD hepatoma 3924A synthase II gene product.

The initial steps of the mammalian de novo uridylate biosynthetic pathway are catalyzed by a multifunctional complex designated CAD (M_r = 210,000) consisting of the first and rate-limiting enzyme, carbamoyl-phosphate synthase II (glutamine-hydrolyzing) as well as the second and third enzymes, respectively, in this pathway, aspartate carbamoyltransferase (EC 2.1.3.2) and dihydro-orotase (EC 3.5.2.3) (5-7). Previous investigations in this laboratory

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The abbreviations used are: BSA, bovine serum albumin; CAD, first three enzymes of de novo UMP synthesis found on a single multienzyme complex, i.e., synthase II, aspartate carbamoyltransferase, dihydro-orotase; kb, kilobases; MOPS, morpholinopropanesulfonic acid; PALA, N-phosphonacetyl-L-aspartate; PEG, polyethylene glycol; synthase II, carbamoyl-phosphate synthase II.

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demonstrated that the activities of these three enzymes of the CAD complex significantly increased and the rise correlated positively with tumor proliferative rates in chemically induced transplantable rat hepatomas (1). The activities were also significantly elevated in rat kidney tumors, sarcomas and colon carcinomas, in human colon carcinoma xenografts and in human primary lung, colon, hepatocellular and renal carcinomas (1,2). Investigations by Reardon and Weber provided evidence that elevated synthase II activity and concentration in the rapidly growing hepatoma 3924A compared to those in liver were due primarily to an increased rate of synthesis in the presence of an unaltered degradation rate (3,4). To elucidate the mechanism of increased tumor synthase II activity, amount, and synthetic rate, rat hepatoma 3924A and liver mRNAs were isolated and the levels of hybridizable CAD mRNA were compared and quantitated by Northern transfer and dot blot analysis. Southern and DNA dot blots were used to investigate the degree of CAD gene amplification in rat hepatoma 3924A compared to that in normal liver.

METHODS

Biological Systems: Chemically-induced, rapidly proliferating hepatoma 3924A was maintained as a bilateral s.c. implant in male ACI/N rats. Tumor proliferation rate was measured in weeks required between inoculation and growth to a diameter of 1.5 - 2.0 cm. Livers of normal rats of the same sex, weight and strain were used as controls.

Poly $(A)^+$RNA Isolation: Total RNA was prepared from rat hepatoma 3924A and liver by extraction with 4 M quanidinium isothiocyanate (Eastman) and centrifugation through 5.7 M CsCl as described by Chirgwin et al. (8). Poly $(A)^+$RNA was obtained by affinity oligo(dT)-cellulose chromatography (second pass) (Pharmacia, Type 7) (9).

DNA Isolation: After being ground to a fine powder in liquid N_2, the tissue powders were dissolved in extraction buffer (0.5 M EDTA, 0.5% sarkosyl, 100 ug/ml freshly prepared protease K) and incubated for 3 h at 50 °C with gentle shaking. The DNA was then extracted (twice) with Tris equilibrated phenol followed by chloroform: isoamylalcohol (24:1), precipitated with 0.1 volume 5 M ammonium acetate (pH = 4.5) and 10 volumes ethanol and then spooled at 4 °C. After resuspending the DNA in TE buffer (10 mM Tris, 1 mM EDTA, pH = 8.0) and adding RNase A (100 ug/ml), the DNA was extracted, precipitated and spooled a second time as described above (10).

Synthetic Oligonucleotide Probe: A 51mer oligonucleotide was synthesized using an Applied Biosystem DNA synthesizer (Model 380A) and 5'-end labeled with [gamma-^{32}P]ATP (7000 Ci/mmol) (New England Nuclear) by T4 polynucleotide kinase (Boehringer Mannheim Biochemicals) (11). This oligonucleotide was made complementary to bases 105-156 of the partial nucleotide sequence of a

recombinant pCAD142, kindly provided by Dr. G. Stark (7). This plasmid is a 6.5 kb cDNA sequenced from the 3' end of a Syrian hamster CAD mRNA constructed in a pBR322 vector utilizing a special simian virus 40 linker from a cloning procedure developed by Okayama and Berg (12).

RNA Blot Analysis: Poly $(A)^+$RNA (15 ug) was electrophoresed on a 0.8% agarose, 2.2 M formaldehyde denaturing gel in the presence of 0.2 M MOPS, 50 mM sodium acetate and 5 mM EDTA, pH = 8.0 (13), and was electrophoretically transferred (Bio-Rad trans-blot system) (30 V, 1h; 80 V, 8 h) to a positively charged nylon Nytran membrane (Schleicher & Schuell, 0.45 uM) in TAE buffer (10 mM Tris, 5 mM sodium acetate, 0.5 mM EDTA, pH = 7.8). Dot blots were prepared by diluting aliquots of mRNA with 15X SSC (1X = 0.15 M NaCl, 0.015 M sodium citrate, pH = 7.0) and filtering the RNA over Nytran membrane pre-equilibrated in 20X SSC using a Hybrid-Dot vacuum apparatus (Bethesda Research Laboratories). In either case, the membranes were baked in vacuo for 2 h, 80 °C and then placed in a plastic bag with 0.5 ml/cm^2 prehybridization solution containing; 50% formamide, 0.1% SDS, 5X Denhardt's solution (1X = 0.02% each of Ficoll, polyvinylpyrrolidone, and BSA), 5X SSPE (1X = 0.18 M NaCl, 10 mM $NaPO_4$, pH = 7.7, and 1 mM EDTA), and 0.2 mg/ml sonicated, denatured calf thymus DNA and incubated for 12 h at 42 °C. Hybridizations were carried out for 24 h, 42 °C, in a solution containing; 50% formamide, 0.1% SDS, 2X Denhardt's solution, 5X SSPE, 10% PEG, and 0.2 mg/ml calf thymus DNA plus the [^{32}P]51mer probe (5 x 10^6 cpm/ml, 6 x 10^8 cpm/ug) denatured (100 °C, 10 min) and quickly cooled on ice. The membranes were then washed twice for 15 min at room temperature in 6X SSC/1% SDS and in 1X SSC/1% SDS followed by a final wash at 30 min at 55 °C in 0.1X SSC/1% SDS, dried and exposed to Kodak X-OMAT film with two intensifying screens for 3 days at -80 °C.

DNA Blot Analysis: Genomic DNA (12 ug) was digested to completion with restriction enzyme, EcoR I (80 U/ul), as specified by the manufacturer (Boehringer Mannheim Biochemicals). The digests were then fractionated on a 0.8% agarose gel, denatured by soaking the gel in 0.5 M NaOH, twice, 15 min, followed by neutralization in 1 M Tris HCl, pH = 7.4, twice, 15 min and equilibration in TAE buffer according to Nytran manufacturer (Schleicher & Schuell). DNA was electrophoretically transferred under the same conditions used for the mRNA. Dot blots were prepared by diluting aliquots of DNA with 15X SSC and incubating at 100 °C for 10 min followed by cooling on ice. DNA blots were prehybridized for 12 h, 42 °C in 1% SDS, 10X Denhardt's solution, 6X SSC, and 50 ug/ml sonicated denatured calf thymus DNA. Hybridizations were carried out for 24 h, 42 °C, in a solution containing; 50% formamide, 1% SDS, 6X SSC, 10% PEG and 0.25 mg/ml calf thymus DNA plus labeled probe (5 x 10^6 cpm/ml, 8 x 10^8 cpm/ug), denatured and chilled. The membranes were washed and autoradiographed as described above.

Quantitations were carried out by using an LKB Ultrascan XL laser densitometer and confirmed by fractionation of total RNA on agarose-formaldehyde gels. For both RNA and DNA gels, Hind III digested DNA and Hinf digested pBR322 high and low molecular weight standards were routinely employed.

RESULTS AND DISCUSSION

A synthetic oligonucleotide was used as a probe to quantitate steady-state levels of CAD specific mRNA. Northern blot hybridizations demonstrated that a large single species of poly$(A)^+$RNA was detected in both rat liver and hepatoma 3924A corresponding to a size of 7.9 kb as determined by comparison with total RNA ribosomal bands and DNA molecular weight standards as described in Methods (Fig. 1a). The identification of the CAD mRNA in the rat tissues

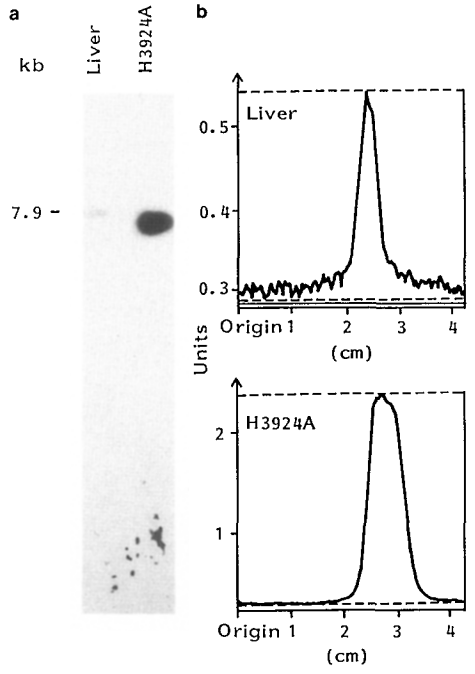

Fig. 1a. Northern blot hybridization of rat liver and hepatoma 3924A poly(A)$^+$RNA and identification of a 7.9 kb CAD mRNA. Fifteen ug of poly(A)$^+$RNA were fractionated according to molecular weight on a 0.8 % formaldehyde agarose gel. Duplicates of each were applied and one lane per sample of liver and hepatoma 3924A poly(A)$^+$RNA, total RNA (liver and tumor) and molecular weight standards (Hind III/λ DNA), (Hinf/pBR322) were stained with ethidium bromide (5 mg/ml) and photographed. Separately, in the unstained portion of the gel, the RNA was electrophoretically transferred to a Nytran membrane and hybridized with the [^{32}P]51mer probe (6 x 10^8 cpm/ug, 5 x 10^6 cpm/ml). Hybridizations were carried out in the presence of 10% PEG for 24 h at 42 ^{o}C as described in Methods. The washed membrane was then exposed to Kodak X-OMAT film with two intensifing screens for 3 days at -80 ^{o}C.

Fig. 1b. A densitometric scan of the autoradiograph produced from Northern blots of poly(A)$^+$RNA liver and hepatoma 3924A. The relative amounts of the CAD 7.9 kb mRNA were estimated from the Northern autoradiograph. (Fig. 1a.)

agreed with previous investigations which had shown the CAD monomer in Syrian hamster cells to be encoded by a 7.9 kb mRNA capable of an *in vitro* translation of a full length CAD protein (6,14). However, no 10.2 kb RNA was detected in our studies in contrast to the identification of this RNA species in the PALA-

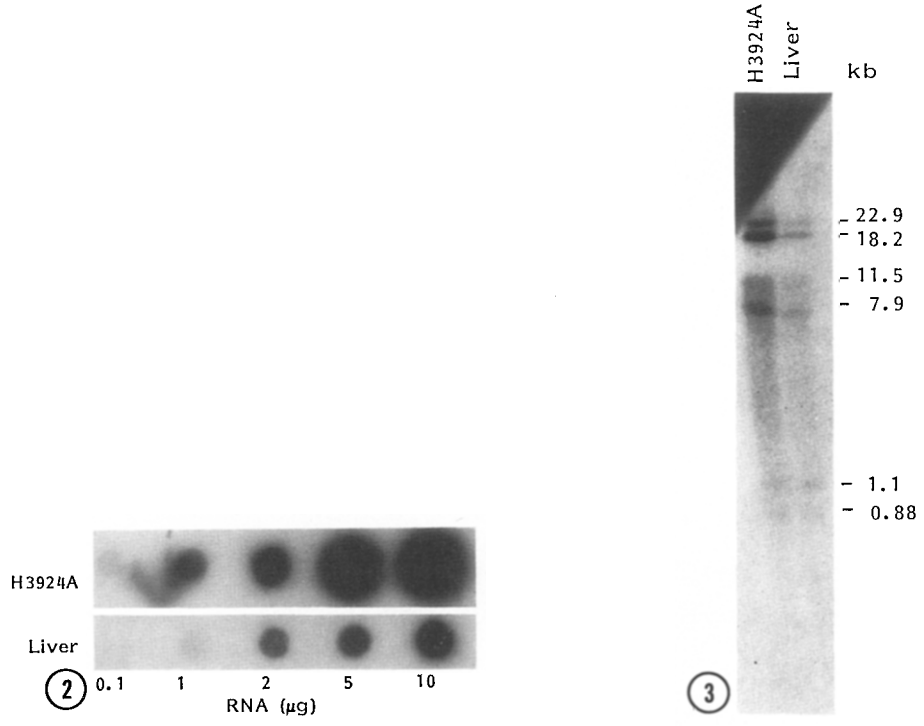

Fig. 2. Comparison of the relative levels of rat liver and hepatoma 3924A poly $(A)^+$ RNA by dot blot hybridization. The poly $(A)^+$RNA for both tissues in each dilution (10, 5, 2, 1 and 0.1 ug) were applied to a Nytran membrane using a Hybrid-Dot apparatus. The dot blot hybridizations were carried out as outlined in Methods. The autoradiographs were quantitated by densitometric scanning and comparison of the relative peak areas obtained from the scans and from the slopes of the lines. (DNA blotted versus amount bound).

resistant Syrian hamster mutants (15). From the autoradiograph (Fig. 1a), it is apparent that the tumor hybridization was markedly increased with respect to the corresponding band generated from liver CAD poly$(A)^+$RNA. Quantitation of these lanes by densitometry showed that the synthase II poly$(A)^+$RNA was 13-fold more abundant in the hepatoma 3924A compared to normal liver (peak areas; 7.9 vs. 0.61)(Fig. 1b). These results were supported by RNA dot blots where direct quantitation indicated that the hepatoma 3924A CAD mRNA increased 11-fold compared to homologous liver (Fig. 2). This increase in the amount of

hepatoma 3924A CAD mRNA was in line with the elevation in synthase II activity (7.9-fold), immunoreactive concentration (8.2-fold) and in vitro synthetic rate (9.7-fold)(1-4).

Southern transfer of rat liver and hepatoma 3924A genomic DNA digested with endonuclease EcoR I produced a blot pattern of 6 visible bands corresponding to estimated sizes of 22.9, 18.2, 11.5, 7.9, 1.1 and 0.88 kb. However, the degree of hybridization especially to the four higher molecular weight DNA fragments was significantly increased in the hepatoma compared to the liver, indicating CAD gene amplification in this tumor (Fig. 3). Additional evidence was provided by DNA dot blots where densitometric scanning and comparison of the slopes of the lines (DNA blotted versus amount bound) resulted in an 8-fold increase in the extent of hepatoma 3924A DNA hybridization to that of the control liver (Fig. 4).

The evidence provided in this study indicates that in the tumor the level of CAD specific mRNA is increased proportionally with the enzyme amount and synthetic rate. The overproduction of CAD mRNA in hepatoma 3924A compared to the levels in normal liver could be the outcome of a coordinate gene amplification. This interpretation would be compatible with the presence of a homogeneously staining region found in the wild type rat hepatoma 3924A cultured cells (16); however, further work will decide between this and

Fig. 3. Southern blot hybridization of EcoR I digests of rat liver and hepatoma 3924A genomic DNA. Genomic DNA (12 ug), digested with restriction enzyme EcoR I (120 U), was fractionated according to molecular weight on a 0.8% agarose gel. This gel was then denatured in 0.5 M NaOH, and neutralized in 1 M Tris HCl (pH ≈ 7.4) and the DNA electrophoretically transferred to a Nytran membrane and hybridized with the [^{32}P]51mer probe (8 x 10^8 cpm/ug, 5 x 10^6 cpm/ml). Hybridizations were carried out in the presence of 10% PEG for 24 h at 42 °C as described in Methods. The washed membrane was then exposed to Kodak X-OMAT film with two intensifying screens for 3 days at -80 °C. In separate lanes, duplicate samples containing the liver and tumor DNA EcoR I digests and the molecular weight standards (Hind III/λDNA, Hinf/pBR322 DNA) were stained with ethidium bromide (5 mg/ml) and photographed.

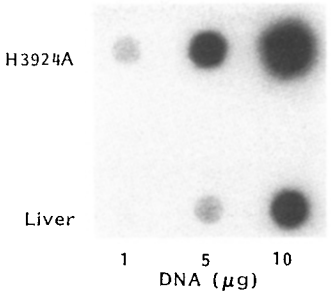

Fig. 4. Comparison of the relative levels of rat liver and hepatoma 3924A genomic DNA by dot blot hybridization. Genomic DNA for both tissues in each dilution (10, 5, and 1 ug) were applied to a Nytran membrane using a Hybrid-Dot apparatus. The dot blot hybridizations were carried out as outlined in Methods. The autoradiographs were quantitated by densitometric scanning and comparison of the relative peak areas obtained from the scans and from the slopes of the lines (DNA blotted versus amount bound).

other possible mechanisms. These results support the proposal that the profound enzymic imbalance observed in this hepatoma is due to a reprogramming of gene expression as suggested by the molecular correlation concept (2).

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