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SELECTION OF A RAT GLUTAMINE SYNTHETASE cDNA CLONE *

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We have selected a glutamine synthetase clone (pGSRK-1) from a rat kidney cDNA library. A partial restriction map has been constructed for the 1.65 kilobase pair (kbp) glutamine synthetase cDNA. Northern hybridization analysis indicates that 1) GS-specific RNA increases many-fold during adipocyte differentiation and 2) dexamethasone increases and insulin decreases GS-specific RNA in 3T3-L1 adipocytes.

Confluent 3T3-L1 cells have the capacity to differentiate into adipocytes (1-5). During the adipocyte conversion of 3T3-L1 cells, glutamine synthetase (GS) specific activity increases by at least 100-fold regardless of the culture conditions employed to accelerate differentiation (8). GS represents a potentially strategic target for the hormonal regulation of nitrogen metabolism because the amide nitrogen of glutamine is utilized for the biosynthesis of wide variety of compounds (14). We reported that GS activity and relative biosynthetic rate are increased by hydrocortisone and decreased by insulin both during

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adipocyte conversion and in fully differentiated 3T3-L1 adipocytes (7,9). In addition, we reported that hydrocortisone increases and insulin decreases the cellular content of translatable GS mRNA in 3T3-L1 adipocytes (10). Recently, multiple-fold amplification of the GS gene has been achieved in subclones of 3T6 cells (16) and Chinese hamster ovary (CHO) cells (13) by selection with the GS inhibitor methionine sulfoximine. An amplified mutant-specific fragment of CHO nuclear DNA was identified and employed to prepare recombinant genomic GS clones (including pGS-103) in the vector pUC-9.

EXPERIMENTAL PROCEDURES

3T3-L1 cells, generously provided by Dr. Howard Green, were grown and maintained as previously described (9). Adipocyte conversion of confluent cultures was achieved by incubation with dexamethasone and 1-methyl-3-isobutylxanthine and insulin for 72 hours according to the method of Rubin et. al. (12). RNA was extracted from cultures with 4M guanidine thiocyanate and isolated by ethanol precipitation. Poly(A+)RNA was isolated by oligo(dT)cellulose chromatography (6). Screening of the rat kidney cDNA library (15) was performed as described (6) except bacterial colonies were grown and screened on 83 mm disks of "GeneScreen" hybridization membrane (New England Nuclear/Dupont). GeneScreen hybridization membranes were pretreated and used with the buffers and under the conditions suggested by the manufacturer. Northern transfers were performed after electrophoretic fractionation of RNA onto a 1.0% agarose gel containing 20 mM MOPS (3-(N-morpholino)-propane sulfonic acid), pH 7, 10 mM sodium acetate and 0.1 mM EDTA (6). Nick-translations, and 5'-end-labeling were performed as described (6). The use of our synthetic tetradecameric oligodeoxyribonucleotide (DNA-85) for hybridization analyses was described previously (10). Prehybridization at 42°C was performed for 6 hours in a solution containing: 40% formamide, 20 mM Pipes (piperazine-N-N'-bis[2-sulfonic acid), pH 6.4, 80 mM NaCl, 0.5% SDS and 20 ug/ml denatured salmon sperm DNA. Hybridization at 42°C was performed for 48 hours under the same conditions as prehybridization but with the addition of nick translated (6) plasmid ($4x10^6$ dpm/ml, $1x10^8$ dpm/ug).

RESULTS AND DISCUSSION

Six putative GS cDNA clones were selected from the rat kidney cDNA library (15). This was accomplished by differential colony hybridization with the nick-translated 2.4 kbp genomic fragment from pGS-103 as the probe. These six were among 100 colonies that had been selected with the previously described synthetic

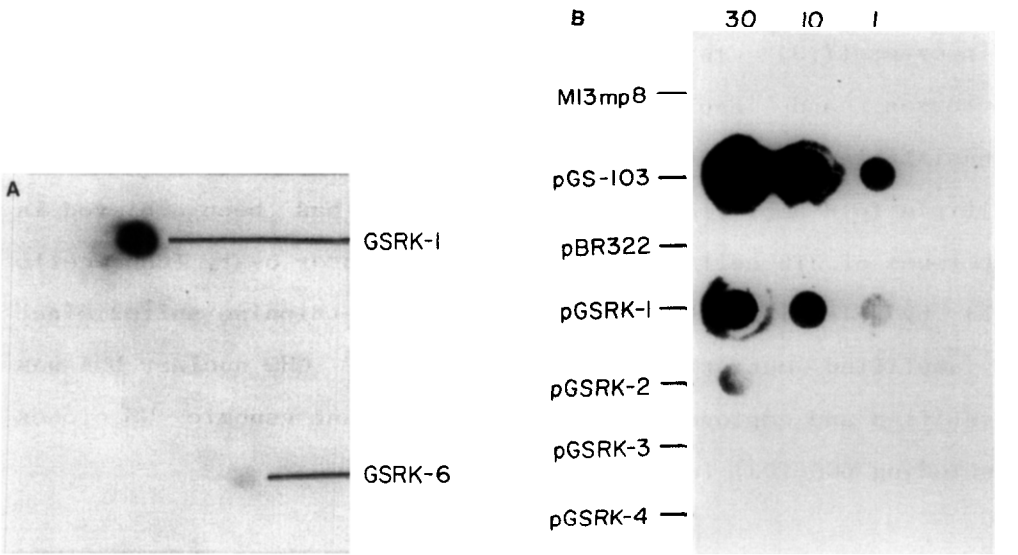

Figure 1. Colony-screening of the rat kidney cDNA library and subsequent rescreening of putative GS cDNA clones. Panel A; Identification of the GS-specific clone GSRK-1 by differential colony-hybridization with the insert from pGS-103 as the probe. The colony designated GSRK-6 proved to be a false-positive. Panel B; Of the 6 clones selected, 4 yielded positive signals. The recombinant plasmid was then isolated from each bacterial clone, quantified by comparision with a standard and dotted in amounts of 1, 10, and 30 ng onto nitrocellulose. M13mp8 and pBR-322 served as the negative controls.

oligonucleotide mixture, DNA-85 (10). The clone yielding the strongest positive signal was designated GSRK-1 (Figure 1, panel A). After colony-purification, only GSRK-1 consistently yielded a strong hybridization signal. Plasmid isolated from clone GSRK-1 and positive (pGS-103) and negative (pBR-322) control plasmids were then rescreened with the ^{32}P-radiolabeled 2.4-kbp GS genomic fragment from pGS-103. Figure 1, panel B indicates that pGSRK-1 hybridizes strongly with the 2.4 kbp pGS-103 fragment whereas pGSRK-2 hybridizes weakly.

pGSRK-1 was isolated by CsCl density gradient sedimentation (6) and characterized by restriction endonuclease digestions. The size of linearized pGSRK-1 is approximately 6 kbp. The plasmid is composed of pBR-322 and a GS cDNA of approximately

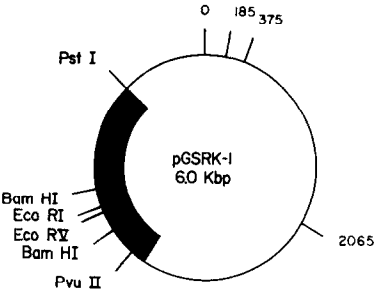

Figure 2. Partial restriction map of pGSRK-1. A partial restriction map of the 1.65 kilobase-pair GScDNA insert in pGSRK-1 is shown. Linearization of the plasmid with Pst I, Hind III or Sal I followed by electrophoretic analyses yielded a length of 6.0 kilobase-pairs. pBR-322 map positions (base-pair) are specified by numerical values.

1.65 kbp. Figure 2 is a partial restriction map for pGSRK-1. In order to be certain of the specificity of our GS cDNA, we analyzed pGSRK-1 hybridizable RNA by the method of Parnes et al (11). The translation of RNA selected with pGSRK-1 yielded a protein comigrating with GS as the only translation product. This translation product was immunoprecipitable with our GS antibody (data not shown).

We performed Northern hybridization analysis of total RNA from both 3T3-L1 adipocytes and rat liver with nick translated pGSRK-1 as the probe. The GS RNA sizes detected in RNA from both sources were identical. With probes of comparable specific radioactivity and optimal hybridization and washing conditions our pGSRK-1 yielded a GS RNA hybridization signal (assessed by autoradiography) which was approximately 10-fold greater than with pGS-103 and 30-fold greater than with DNA-85. Qualitative Northern hybridization analysis (Fig. 3) indicates that GS-specific RNA increases many-fold during adipocyte conversion (compare lanes 1 and 2). In addition, dexamethasone increases (Compare lanes 2 and 3 with lane 4) and insulin decreases

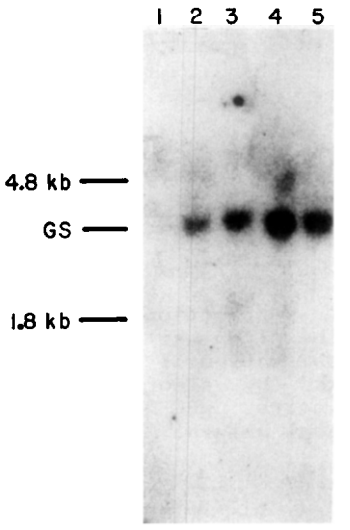

Figure 3. Northern hybridization analysis of total RNA from 3T3-L1 preadipocytes and adipocytes. 3T3-L1 cells were plated (3 x 10^4 cells/cm^2) and mantained with medium containing no added hormones for a time sufficient to achieve confluence (3 days). Confluent monolayers were harvested to obtain preadipocyte RNA (lane 1). Adipocyte differentiation was achieved as follows. First the remaining confluent monolayers were incubated with 1 uM dexamethasone, 0.5 mM 1-methyl-3-isobutylxanthine and 10 ug/ml insulin for 72 hours (days 0 to 3). Next the cultures were incubated for 48 hours with 1 uM dexamethasone and 1 ug/ml insulin (days 4 and 5)to promote adipocyte differentiation. Beginning on day 5 fresh medium was added containing no additions (lanes 2 and 3), 1 uM dexamethasone (lane 4), and both 1 uM dexamethasone and 1 ug/ml insulin (lane 5). After 4 hrs of incubation (except lane 2, zero hour) adipocyte cultures were harvested and RNA was prepared. Total RNA (70 ug) was applied to each lane. The Figure is an autoradiograph of the Northern blot analysed by hybridization with ^{32}P-labeled pGSRK-1.

(Compare lanes 4 and 5) GS-specific RNA in differentiated 3T3-L1 adipocytes.

pGSRK-1 will allow isolation of mouse GS genomic DNA clones. Analysis of these will provide knowledge of the structure, organization and regulatory regions of the GS gene.

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