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Cloning, nucleotide sequence, and potential regulatory elements of the glutamine synthetase gene from murine 3T3-L1 adipocytes

[poly(dT-dG)·poly(dC-dA) element/glucocorticoid response elements/cAMP response elements/fat-specific elements]

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ABSTRACT **Glutamine synthetase [L-glutamate:ammonia ligase (ADP-forming); EC 6.3.1.2] specific activity, cellular content, mRNA abundance, and gene transcription rate increase by >100-fold during adipocyte differentiation of 3T3-L1 cells. In 3T3-L1 adipocytes dexamethasone increases, whereas insulin as well as N^6,$O^{2'}$-dibutyryladenosine 3',5'-cyclic monophosphate decrease, glutamine synthetase gene expression. We analyzed the nucleotide sequence of a 1.9-kilobase *Sal* I–*Eco*RI restriction fragment from a 3T3-L1 glutamine synthetase genomic clone. This genomic fragment is composed of 1851 base pairs (bp) and includes the first exon and 1029 bp of the 5' flanking sequence. The 600 bp at the 3' end of the 1.9-kb *Sal* I–*Eco*RI restriction fragment constitute an open reading frame. We identified the transcription start site at a location 222 bp upstream of the glutamine synthetase coding sequence. The 5' flanking region of the gene encompasses several potential regulatory elements including TATA and CAAT sequences and a 40-bp poly(dT-dG)·poly(dC-dA) putative enhancer element. Potential hormone and fat-specific regulatory elements are also located upstream of the transcription start site; they include glucocorticoid and cAMP response elements and fat-specific elements. These potential regulatory elements could account for the differentiation-associated changes and hormone-mediated changes seen in glutamine synthetase gene transcription and mRNA abundance.**

Glutamine synthetase [L-glutamate:ammonia ligase (ADP-forming); EC 6.3.1.2] specific activity and cellular content increase by >100-fold during adipocyte differentiation of murine 3T3-L1 cells (1–3). In 3T3-L1 adipocytes N^6,$O^{2'}$-dibutyryladenosine 3',5'-cyclic monophosphate (Bt_2cAMP) decreases and hydrocortisone increases glutamine synthetase activity and cellular content (2, 4). Our studies of glutamine synthetase in 3T3-L1 adipocytes indicate the following: (*i*) glutamine synthetase biosynthetic rate is increased by hydrocortisone and decreased by insulin and Bt_2cAMP (2, 3); and (*ii*) glutamine synthetase gene transcription rate increases >100-fold during adipocyte differentiation, and the transcription rate is hormonally regulated in 3T3-L1 adipocytes. Dexamethasone increases glutamine synthetase mRNA amounts and glutamine synthetase gene transcription, whereas both insulin and Bt_2cAMP prevent these increases (4, 5). To study the mechanisms for the differentiation-associated and hormone-mediated changes in glutamine synthetase gene expression we isolated the glutamine synthetase gene from 3T3-L1 cells and analyzed the nucleotide sequence of the first exon and >1000 base pairs (bp) of the 5'-flanking sequence.*

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MATERIALS AND METHODS

Materials. T7 DNA polymerase and dideoxynucleotide triphosphates were from United States Biochemical (Cleveland). Deoxyadenosine [α-^{35}S]thiotriphosphate (600 Ci/mmol; 1 Ci = 37 GBq), [γ-^{32}P]dATP (3000 Ci/mmol), and [α-^{32}P]dCTP (3000 Ci/mmol) were from Amersham. Avian myoblastosis virus reverse transcriptase was provided by J. Leis (Case Western Reserve University) (6). EMBL-3 arms and λ packaging extract were from Stratagene (La Jolla, CA). Ribonuclease inhibitor was from Promega Biotec (Madison, WI).

Glutamine Synthetase Genomic Clones. We prepared two separate 3T3-L1 genomic libraries in the λ replacement vector EMBL-3 (7, 8). Genomic DNA was isolated (8, 9) from 3T3-L1 preadipocytes, partially digested with *Sau*3A, size-fractionated by sucrose density-gradient centrifugation, ligated to EMBL-3 arms and packaged. Approximately 6×10^5 plaques were screened with ^{32}P-labeled pGSRK-1 (glutamine synthetase cDNA) as probe (10).

Nucleotide Sequence Analysis. The 1.9-kb *Sal* I–*Eco*RI restriction fragment (*SE*-1.9) of our glutamine synthetase genomic clone was subcloned into vector M13mp18/19 (11) and further truncated by sequential deletion with Bal-31 exonuclease (12). Nucleotide sequence was analyzed by the dideoxynucleotide chain-termination method (13) with T7 DNA polymerase (14). Initial sequence data were obtained with an M13 universal primer. In addition, we prepared five synthetic oligonucleotide primers (Figs. 2 and 3).

Primer-Extension Analysis of Glutamine Synthetase mRNA. The oligonucleotide primer, complementary to nucleotides 1069–1093 (Fig. 2), was 5' end-labeled with T4 polynucleotide kinase (15). Hybridizing of primer and RNA was achieved by heating hybridizing mixtures to 65°C for 3 min and then slowly cooling them to 42°C. Hybridizing mixtures contained 20 mM Tris·HCl (pH 8.3), 500 mM KCl, 10 ng (3.5×10^6 dpm) of radiolabeled primer, and 50 μg of total RNA from 3T3-L1 adipocytes (16) or 10 μg of wheat germ tRNA in a final volume of 10 μl. Primer-extension reaction mixtures, incubated at 42°C for 45 min, contained 50 mM Tris·HCl (pH 8.3), 10 mM $MgCl_2$, 125 mM KCl, 5 mM dithiothreitol, 1 mM of each dNTP, 50 units of avian myoblastosis virus reverse transcriptase, 10 units of RNase inhibitor, and 10 ng of ^{32}P-labeled primer hybridized with 50 μg of 3T3 adipocyte total RNA or 10 μg of wheat germ tRNA in a final volume of

Abbreviations: CHO, Chinese hamster ovary; *SE*-1.9, the *Sal* I–*Eco*RI restriction fragment of the 3T3-L1 GS genomic clone; nt, nucleotide(s); GRE, glucocorticoid response element; CRE, cAMP response element; FSE-1 and FSE-2, fat-specific elements 1 and 2, respectively; ORF, open reading frame.

*The sequence reported in this paper is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03820).

40 μl. Primer-extension products were analyzed by electrophoresis on a 6% polyacrylamide sequencing gel.

S1 Nuclease Protection Analysis. The DNA probe used for S1 analysis was prepared by extension of the oligonucleotide primer corresponding to nucleotides (nt) 1252–1276 (Fig. 3). The radiolabeled extension products were digested with *Hin*dIII (restriction site at nt 682, Fig. 2) to yield a single-stranded probe of 595 nt. This radiolabeled probe was incubated (to allow hybridizing) with 50 μg of total RNA from 3T3-L1 adipocytes or with 10 μg of wheat germ tRNA. After hybridizing, reaction mixtures were digested with S1 nuclease (17, 18), and the digestion products were analyzed as described for primer extension.

RESULTS

Glutamine Synthetase Genomic Clones. We identified three glutamine synthetase clones in our 3T3-L1 genomic libraries. All three clones yielded the same restriction map with three restriction endonucleases. *Eco*RI digestion yielded four fragments [7.6, 2.7, 1.3, and 4.5 kilobases (kb) in 5′ to 3′ order] in addition to the EMBL-3 arms. *Bam*HI digestion yielded two restriction fragments (8.4 and 7.6 kb) and *Sal* I digestion also yielded two fragments (6.0 and 11.9 kb). Simultaneous digestion with pairs of these restriction enzymes in combination with the results from single restriction enzyme digestions yielded the restriction map in Fig. 1. Fragments that hybridized with pGSRK-1 were 1.9, 1.3, and 0.4 kb in length.

Glutamine Synthetase Coding Sequence. The nucleotide sequence data (Figs. 2 and 3) are for the 1.9-kb *Sal* I–*Eco*RI fragment (*SE*-1.9) mapped in Fig. 1. The sequence for the entire *SE*-1.9 restriction fragment is composed of 1851 nt (Figs. 2 and 3). The 600 nt at the 3′ end of *SE*-1.9, beginning at nt 1252, constitute an open reading frame (ORF) of 200 amino acids.

Putative Regulatory Elements of the Glutamine Synthetase Gene. The 1029-bp sequence at the 5′ end of *SE*-1.9 includes several potential regulatory elements (Fig. 2). Probable locations of functional TATA and CAAT sequences are at nt 1001 and 918 (19). We identified a 40-bp poly(dT-dG)·poly(dC-dA) putative enhancer element (20, 21) 265 bp upstream of the transcription start site (Figs. 2 and 4). We also identified potential hormone-response and fat-specific regulatory elements in the 5′ flank of the gene. These elements closely resemble already identified regulatory sequences of other genes, which include the glucocorticoid response element (GRE), the cAMP response element (CRE), and fat-specific elements types 1 and 2 (FSE-1 and -2) (Fig. 2).

Sequences having 100% identity with the octanucleotide core GRE, AGAWCAGW (where W = A or T) (22), occur in the direct orientation at nt 433 and 568 (Fig. 2). Sequences having 87.5% identity with this GRE occur in the direct orientation at nt 138, 145, 428, 535, and 560, and in the inverted orientation (5′ → 3′ on the complementary DNA strand) at nt 163 and 890.

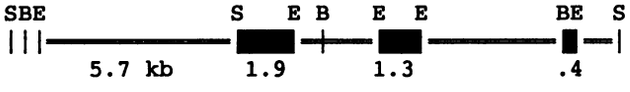

FIG. 1. Restriction endonuclease map of a 3T3-L1 glutamine synthetase genomic clone in EMBL-3. Glutamine synthetase genomic DNA in EMBL-3 was digested with the indicated restriction endonucleases. S, *Sal* I; B, *Bam*HI; and E, *Eco*RI. Digestion mixtures were subjected to agarose gel electrophoresis and Southern hybridization analysis. Restriction fragments that hybridized with the radiolabeled glutamine synthetase cDNA probe (pGSRK-1) are indicated by solid bars; numbers beneath bars indicate kb. The 5′ → 3′ orientation is left to right.

One sequence in the direct orientation at nt 401 has 100% identity with the 7-bp core consensus sequence (TGMGTCA, where M = A or C) of a CRE (23, 24). Ten other 8-bp sequences, six direct (at nt 390, 400, 456, 474, 659, and 926) and four inverted (at 397, 463, 547, and 933), have 75% identity with a similar octanucleotide CRE consensus sequence (TKACGTCA, where K = G or T). Three sets of these sequences (390 and 397, 456 and 463, 926 and 933) are complementary pairs.

Sequences having 69% identity with the 13-bp FSE-1 consensus sequence (GGCWCTGGTCAKG) (25) occur in the direct orientation at nt 158, 329, and 656 and in the inverted orientation at nt 47 and 563. Sequences having at least 71% identity with specific FSE-1 sequences (in parentheses) (25) occur in the direct orientation at nt 44 (AGCACTGTCAGC), 158 (TACTCTGTTCATG) and 453 (GGCTGAGGCCAGG), and in the inverted orientation at 569, 857 (TCCTCTGAGTCATG), and 890 (AGCACTGTCAGC). Sequences having at least 69% identity with the FSE-2 consensus sequence (ACTCRGAGGAAAA, where R = G or A) (26) occur in the direct orientation at nt 477, 561, and 704, and in the inverted orientation at 202, 346, 746, and 878.

Three pairs of 10-bp tandem repeats (TR) appear in the 5′ flank (Fig. 2); these occur at nt 70 and 841 (TR-1), 421 and 528 (TR-2), and 483 and 612 (TR-3).

Transcription Start Site of the Glutamine Synthetase Gene. To identify the transcription start site we estimated the 5′ extent of glutamine synthetase mRNA by primer extension. During incubation of the 5′ end-labeled primer with total RNA from 3T3-L1 adipocytes and avian myoblastosis virus reverse transcriptase, the primer was extended to an upstream location corresponding to nt 1030. No extension of the primer occurred when wheat germ tRNA was substituted for 3T3-L1 RNA. We confirmed the location of the transcription start site by S1 nuclease protection analysis of a probe that spanned the putative start site. The portion of the probe protected by hybridizing with total RNA from 3T3 adipocytes corresponded to a transcription start site at nt 1026–1030 (Fig. 2).

DISCUSSION

The nucleotide sequence for 1029 bp of the 5′ flank and for exon one of the glutamine synthetase gene from 3T3-L1 cells is particularly relevant because the glutamine synthetase gene in 3T3-L1 cells is subject to developmental as well as multihormonal control (1–3). Therefore, analysis of glutamine synthetase gene expression has the potential to help unravel the mechanisms by which development and hormones regulate gene expression. To our knowledge the glutamine synthetase gene in 3T3-L1 adipocytes is the only glutamine synthetase gene subject to multihormonal control. Only one other eukaryotic glutamine synthetase gene has been sequenced, the one from the plant alfalfa (27).

Between nt 1 and 1252 of *SE*-1.9 there are no ORFs longer than 165 nt. Between nt 1252 and 1851 are two long ORFs. One extends from nt 1252–1851. For this 600-bp ORF the identity between 3T3-L1 glutamine synthetase and methionine sulfoximine-resistant CHO glutamine synthetase (28, 29) is 90% for the nucleotide sequence and 93% for the corresponding deduced amino acid sequence. Amino acid 45 and nt 1384–1386 in the 3T3-L1 glutamine synthetase genomic sequence do not occur in the CHO glutamine synthetase cDNA sequence (Fig. 3, *). Between amino acids 91 and 92 in the 3T3-L1 sequence are three amino acids in the CHO sequence (arginine, aspartic acid, and proline) that do not occur in the 3T3-L1 sequence (Fig. 3, **). The other relatively long ORF (231 bp) is from nt 1352–1582. The deduced amino acid sequence for this ORF has little, if any, identity with that for the glutamine synthetase cDNAs from CHO

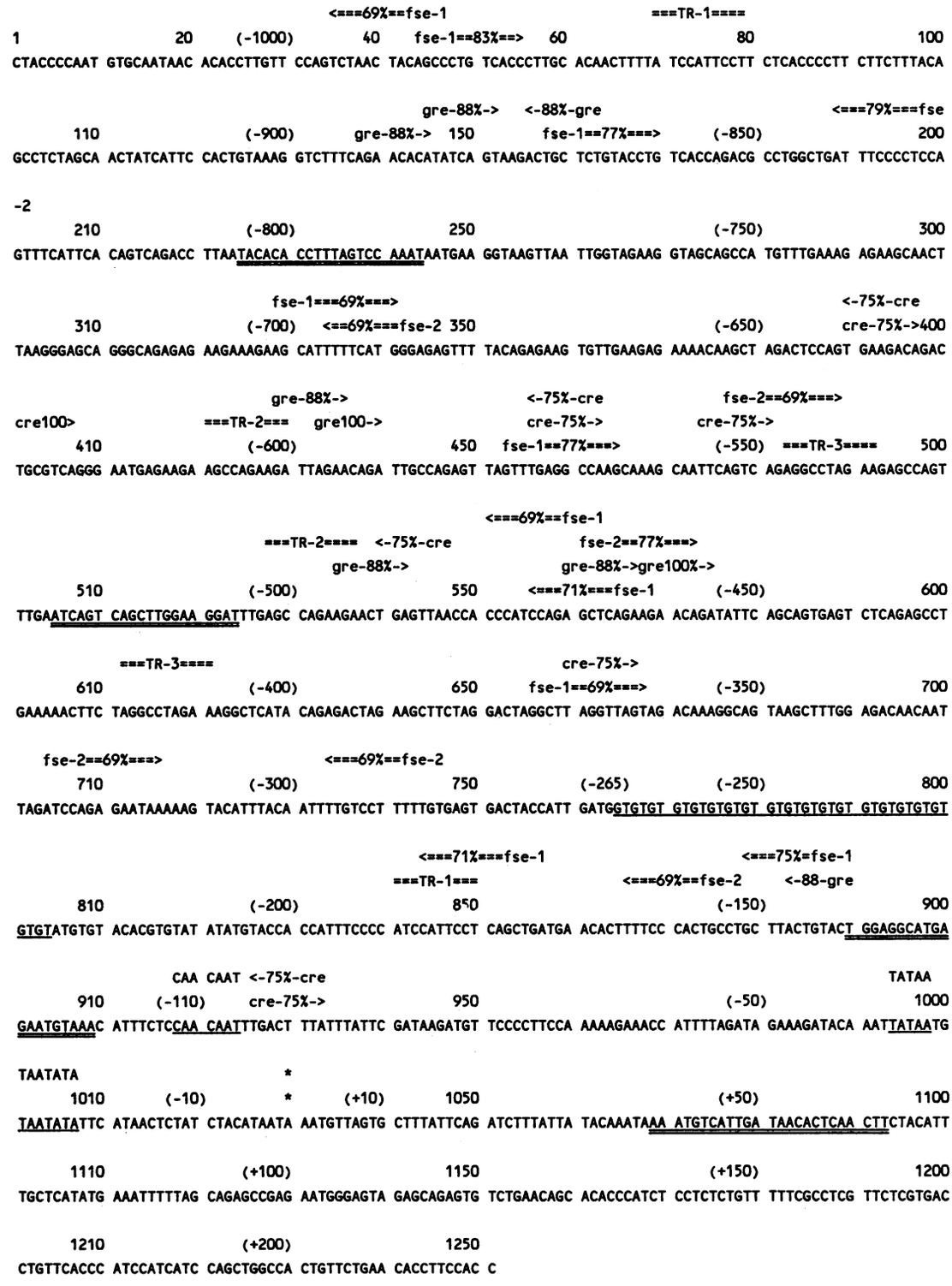

FIG. 2. Nucleotide sequence upstream from the first glutamine synthetase codon. Putative regulatory elements are designated above the nucleotide sequence as follows: GRE, gre-%->; CRE, cre-%->; FSE-1 (fat-specific element 1), fse-1== %===>; FSE-2 (fat-specific element 2), fse-2== %===>. %, Percent identity with the sequence indicated in text. The arrowhead designates the 5′ → 3′ orientation. Nucleotides are numbered above sequence starting with the 5′ end of *SE*-1.9 and in parentheses starting with the transcription start site (+1). TR-1, TR-2, and TR-3 indicate the location of three pairs of 10-bp tandem repeats. The vertical double asterisks at nt 1030 indicate the probable transcription start site. Single-underlined sequences are the $(TG)_{20}$ element and potential TATA and CAAT sequences. Double-underlined sequences are those for which synthetic oligonucleotide primers were prepared (direct for nt 225, 505, and 890; inverted for nt 1069 and 1252).

cells (29) or human liver (30). These data indicate that the correct ORF for 3T3-L1 glutamine synthetase begins at nt 1252 and extends through the 3′ end of *SE*-1.9.

As noted above a very high degree of identity exists between the deduced amino acid sequence for 3T3-L1 glutamine synthetase and that for glutamine synthetase from methionine sulfoximine-resistant CHO cells. In addition, the 144-bp sequence in *SE*-1.9 upstream of the initiator ATG has 67% identity with the corresponding sequence in the glutamine synthetase cDNA from the CHO cells (29). This sequence similarity is even greater for shorter segments. Moreover, we reported that glucocorticoids increase tran-

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   (1)    1260                        1280                        1300         (20)        1320
1) ATG GCT ACC TCA GCA AGT TCC CAC GTG AAC AAA GGC ATC AAG CAA ATG TAC ATG TCC CTG CCC CAG GGT GAG AAA ATC CAA
2) M   A   T   S   A   S   S   H   Vl  N   K   Gn  I   K   Q   M   Y   Ml  Sc  L   P   Q   G   E   K   Iv  Q
3)       C                       T           AA                      T   G                       G
         1340                        1360          (40)        1380                        1400
1) GCC ATG TAT ATC TGG GTT GAT GGT ACC GGA GAA CCA CTG CGC TGC AAG ACC TGT CGT ACC CTG GAC TGT GAG CCC AAG TGT
2) A   M   Y   I   W   V   N   G   T   G   E   P   L   R   C   K   T   C*  R   T   L   D   C   E   P   K   C
3)                                   T
       1420          (60)        1440                        1460                        1480          (80)
1) GTG GAA GAG TTA CCT GAG TGG AAC TTT GAT GGC TCC AGT ACC TTT CAG TCT GAA GGC TCC AAC AGC AAC ATG TAT CTC CAT
2) V   E   E   L   P   E   W   N   F   D   G   S   S   T   F   Q   S   E   G   S   N   S   Nd  M   Y   L   Hs
3)                           T               T                       G               T G                 AGC
     1500                        1520                        1540          (100)       1560
1) CCT GTT GCC ATG TTT AGA GAC CCC TTC CGC AAC AAG CTG GTG CTA TGT GAA GTT TTC AAG TAT AAC CGG AAG CCT GCA GAG
2) P   V   A   M   F   R   D   P   F   R   N   K   L   V   Lf  C   E   V   F   K   Y   N   R   K   P   A   E
3)                   C G             **                  T C                 C
    1580                        1600          (120)       1620                        1640
1) ACC AAC TTG AGG CAC ATC TGT AAA CGG ATA ATG GAC ATG GTG AGC AAC CAG CAC CCC TGG TTT GGA ATG GAG CAG GAA TAT
2) T   N   L   R   H   Is  C   K   R   I   M   D   M   V   S   N   Q   H   P   W   F   G   M   E   Q   E   Y
3)       T   A       TCG                                                                         A       G
   1660          (140)       1680                        1700                        1720          (160)
1) ACT CTC ATG GGA ACA GAC GGC CAC CCG TTT GGT TGG CCT TTC AAT GGC TTC CCT GGA CCC CAA GGC CCA TAT TAC TGC GGT
2) T   L   M   G   T   D   G   H   P   F   G   W   P   Fs  N   G   F   P   G   P   Q   G   P   Y   Y   C   G
3)       G               T   G       T               C           T       G           T   G           T
  1740                        1760                        1780          (180)       1800
1) GTG GGA GCA GAC AAA GCC TAT GGC AGG GAC ATC GTG GAG GCT CAC TAC CGG GCC TGC TTG TAT GCC GGA GTC AAG ATC ACG
2) V   G   A   D   K   A   Y   G   R   D   I   V   E   A   H   Y   R   A   C   L   Y   A   G   V   K   I   T
3)       C                           T                           C               T   G           T
 1820                        1840          (200)
1) GGG ACA AAT GCG GAG GTT ATG CCT GCC CAG TGG
2) G   T   N   A   E   V   M   P   A   Q   W
3)   A           T       C
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FIG. 3. Coding sequence of *SE*-1.9. Line 1 represents the sequence of 600 nt at the 3′ end of *SE*-1.9. Line 2 represents the deduced amino acid sequence (single-letter code; uppercase letters) and differences between the deduced amino acid sequence for 3T3-L1 glutamine synthetase and that for Chinese hamster ovary (CHO) glutamine synthetase (lowercase letters). Line 3 indicates the differences between the 3T3-L1 nucleotide sequence and that for the coding sequence of the glutamine synthetase cDNA from methionine sulfoximine-resistant CHO cells (29). Numbers above the nucleotide sequence indicate amino acid number (in parentheses) and nucleotide number (starting at the 5′ end of *SE*-1.9). The double-underlined sequence (1276–1252) served for synthesis of a complementary synthetic oligonucleotide. *, Amino acid 45 and nt 1384–1386 in the 3T3-L1 sequence do not occur in the CHO sequence. **, Three amino acids in the CHO sequence that do not occur in the 3T3-L1 sequence.

scription of the amplified glutamine synthetase gene in the methionine sulfoximine-resistant CHO cells (31). These observations indicate that there might be segments of considerable similarity between 5′-flanking sequences of the glutamine synthetase gene in CHO cells and in 3T3-L1 cells.

Note that the sequence around the initiator ATG codon at nt 1252 (CCACCATGG) (Figs. 2 and 3) is identical to the optimal sequence for initiation by eukaryotic ribosomes (32). Moreover, the glutamine synthetase gene is unusual in that there are two other ATG triplets upstream of the initiator ATG, located at nt 1032 (out of frame with the initiator ATG) and at 1108 (in frame). Neither of these is flanked by the optimal sequence for initiation of protein synthesis. Furthermore, both are in frame with TGA terminator codons at nt 1197 and 1153.

The relatively minor differences between the coding region of the glutamine synthetase gene from 3T3-L1 cells (derived from Swiss mouse) and the glutamine synthetase cDNA from CHO cells are attributable, at least in part, to species differences. The CHO cDNA sequence is from methionine sulfoximine-resistant CHO mutants with multiple copies of an amplified glutamine synthetase gene (28, 29). Therefore, despite similarities between coding and small noncoding segments of the 3T3-L1 glutamine synthetase gene and the CHO glutamine synthetase cDNA, there is potential for considerable nonidentity between glutamine synthetase genomic flanking sequences from the two cell types.

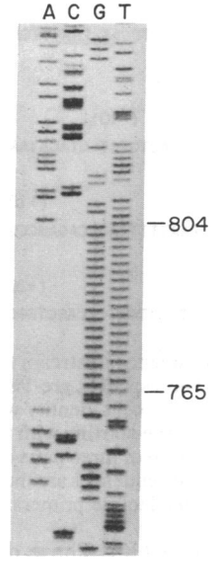

FIG. 4. The $(TG)_{20}$ element in the *SE*-1.9 fragment of the glutamine synthetase gene. Autoradiograph of a portion of a sequencing gel that demonstrates the $(TG)_{20}$ element in the *SE*-1.9 fragment. The sequencing primer corresponded to nt 505–524 in Fig. 2. Numbers at right indicate nucleotide location in Fig. 2.

Complete or partial amino acid sequence data (deduced or obtained directly) are available for glutamine synthetase from a few eukaryotic species (27, 29, 30, 33–35). The deduced, 13-amino acid 3T3-L1 glutamine synthetase peptide from amino acids 155–167 (Fig. 3) has 100% identity with the *Saccharomyces cerevisiae* glutamine synthetase cysteine-containing peptide from amino acids 156–168 (table 3 of ref. 35). By contrast, there is no similarity between the deduced N-terminal octapeptide of 3T3-L1 glutamine synthetase and that of *S. cerevisiae* glutamine synthetase (35). Our analysis of 3T3-L1 glutamine synthetase together with the detailed analyses of glutamine synthetase from CHO cells (29), alfalfa (27), and yeast (35) indicate substantial similarity between certain segments of glutamine synthetase from mammals, yeast, and plants.

We identified a 40-bp poly(dT-dG)·poly(dC-dA) sequence, and several potential hormone-response and fat-specific elements in the 5′ flank of the glutamine synthetase gene. In addition, we identified the location of the transcription start site (nt 1030) and probable locations for the TATA and CAAT boxes. Several potential TATA boxes exist in close upstream proximity to nt 1030. Two of these are located at nt 994 (TATAAT) and 1001 (TAATATA). Although both sequences resemble the consensus TATA sequence (TATAWAW) only the one at nt 1001 is the expected distance upstream of the transcription start site (26–34 bp) (19). At least one potential CAAT box is located in close upstream proximity of the putative transcription start site; it is located at nt 918 (CAACAAT), which is further upstream (−112 bp) than expected (−70 to −80 bp) (19). Definitive location of TATA and CAAT boxes requires functional studies.

Poly(dT-dG)·poly(dC-dA) elements have been seen associated with the coding sequences (in the 5′ flank or in introns) of a few cloned genes (20, 21, 36). Moreover, these poly(TG) elements exhibit enhancer activity when inserted upstream of a viral promoter. Thus, the poly(TG) element could function in differentiation-associated and/or hormone-mediated regulation of glutamine synthetase gene expression.

We have identified several putative regulatory elements in the 5′ flank of the glutamine synthetase gene from 3T3-L1 cells. These include poly(dT-dG)·poly(dC-dA), GRE, CRE, FSE, and tandem-repeat elements. Although functional analyses are required to establish the roles of these sequences in glutamine synthetase regulation, their presence is consistent with observed regulation of glutamine synthetase during adipocyte differentiation and in 3T3-L1 adipocytes (1–5). Assessment of the functional activity of putative regulatory sequences will require analysis of 3T3-L1 cells transfected with chimeric plasmids that include potential regulatory elements, the glutamine synthetase promoter, and a reporter gene. In addition, DNase I footprint analyses of the glutamine synthetase gene and its flanking sequences should locate the functional regulatory elements.

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