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Promoter paper

Molecular cloning of the rat TA1/LAT-1/CD98 light chain gene promoter¹

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Abstract

The rat LAT-1 (L-amino acid transporter-1) gene is a CD98 light chain highly expressed in cancer and development. As an initial study of the molecular basis underlying regulation of its expression, we cloned 2 kb of the LAT-1 5' flanking region. Inverse RACE and primer extension methods were used to define the transcription initiation site at 80 bp upstream from the translational start site. Functional studies carried out in normal hepatic cells using constructs containing progressive 5' deletion from region –1958 to –185 showed 3–5-fold β -galactosidase activities over control. The presence of an activator site(s) between –52 and –185 was indicated by low activities conferred by the construct spanning this region. © 2001 Elsevier Science B.V. All rights reserved.

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TA1 (tumor antigen 1)/LAT-1 (L-type amino acid transporter), whose gene was cloned in our laboratory [1] and others' [2], is a recently characterized light chain of the CD98 complex [3] and a member of the newly described and growing family of membrane-associated glycoprotein amino acid transporters [4]. The CD98 complex, which consists of 4F2 heavy chain and a light chain isoform, has been ascribed important functions in a variety of cellular processes including lymphocyte activation, integrin signaling, membrane fusion and amino acid transport [5–8]. We have demonstrated TA1 overexpression in rat hepatocellular carcinoma, human colon cancer, and other primary neoplasias [1,9]. In addition, transient, immediate upregulation of TA1 was observed in liver regeneration after partial hepatectomy and toxic injury [10]. Involvement of CD98 light chain in tumor progression is strongly suggested by a recent study which showed that upregulation of the CD98 complex, but not the CD98 heavy chain alone, in Balb3T3 cells resulted in tumorigenicity in nude mice [11].

Recently, we reported that the level of TA1/LAT-1

RNA expression in primary hepatocytes and non-transformed, immortalized hepatic cells can be modulated by amino acid (arginine) availability [12]. Intriguingly, availability of arginine also affected amino acid transport properties in these cells, but not in transformed and tumorigenic hepatic lines [12]. These data indicate that hepatic TA1/LAT-1 expression is normally under tight regulation. However, little is known in terms of the mechanisms underlying the regulation of TA1/LAT-1. Indeed, information regarding molecular regulation of amino acid transporters or mechanisms underlying the adaptive response of eukaryotic genes to amino acids in the nutrient environment is generally lacking [13]. Exceptions are the known transcriptional regulation of mCAT-2 (murine cationic amino acid transporter-2) [14,15] and the transcriptional regulation of a well-described amino acid responsive gene, asparagine synthetase [16]. Two reports have described an amino acid responsive nucleic acid sequence element in the amino acid-responsive gene asparagine synthetase [16,17] and, very recently, a similar element was defined in CHOP [18], suggesting a novel mechanism of adaptive transcriptional regulation.

To gain insight into the molecular mechanisms underlying the regulation of TA1/LAT-1 expression, the LAT-1 gene promoter was isolated. An approx. 2 kb fragment of the 5' flanking region of LAT-1 gene was cloned from a

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¹ GenBank accession No. AF329652.

-1958 AATTCCTGG CACCGACCT CCCAGCTGC CCAGATCACA GGTCCCCCTC TGCTTTCCTT GTTCATTCTT
 -1888 TCCTTAATT CCAGACGCAG AAAAGCCATT TGAATCTGCT GCGCTCCTCC ACGGCCACCC CTTTGGGCT
 -1818 CTAGTAGTGG CCTTGGTTTA ATTGTGGTTG TGATTTTACA CCTCCGTTGA CTATTGCTGT CTCTGGCTCA
 -1748 AATCAAAGCT TCCCCACAGC GGGTATGCAG AACCTAGCCA CTGTGAATGG GGACGGATTCC CAAAAGAAGC
 -1678 AAGGGTTAGC CACCCACAGG TGAGAGGTGA GCAGGTTGCT TGACTTGAGG TAGGAAGCCA CGAGGGTGCA
 -1608 CATACTGGGC AGGCAAGTTA CACAAGTCT AAGTTCGGTTT CCTCACAAAG TGTTTCTAAG GGTCGCCACG
 -1538 TGTCCTATTA GGTTAGGCGC TGTACCTTGG AATCTTAGCA ATGTCTCAGT GGGTTGGTGG TATGCTTTTA
 -1468 GCCCTCCGA AGGACCGTGT AAAGAATGAGAGGCA AAGTTT CTCAATCTTC CCAAAGCCAC CAATTTAGGC
 -1398 AGTCTGTAGC ATGGCATGTT AATGTCGTGA CCTTTAGCT CCATAGCTTC CCACCGCCCA GGCTACAGAG
 -1328 ACTCCGGCTT GTCATGGCTT GTCACTGTGT TTGAATGGCT GACATACTGG AGTGGGGGATGTGGGTAGG
 -1258 GAGAGGTGACTCCAGTGACC CTTGGAGTCC ATGGCTGAAC ACCCCCCTCC CCGACGATGT GGGCTCCAC
 -1188 GGACGGCTCT GGCATCTTGT GCAAAAAATA TGGATCCAGA GAAGGCGAGGGGTTGAATT TCTTCATTGG
 -1118 TCATGCCACA CGAGTGGGAA AGACCTGGCA GACTGGGTTT TGCAGAGCAC ACAGGGTITT GCATCCCTCT
 -1048 GAGTCTGAAG GTTCTTCTTG AATTAAGCCC CTCACCCCTCC CTTCTGCTTG TCCGTGAGT TFCCTGGGGA
 -978 CCCCCTTGGG TGAAAAAGGGAACAGTCAAG TGAAAAAGGAGAGAGTCTGG GGCTTCTGG TCTGACCTTG
 -908 ACTGATGAAACTTTCTAAGC ACGACTGTCA GCTCAAATCA CCATGCCAGG CAACCAGGCC TGAATGTTC
 -838 AATCTTTCAT CATTCCTC AAAGCCATC TCACCTAAGA CTTCTAAGA ATGACAAACT AAGGTCTTT
 -768 CACCTCAGCCT GCTTTTCAAATCCCTATGTA GATAGGACAGTTCCTCTCT TCTAAAGCTT GCTCCTCCAG
 -698 CTGGGTGTA CCACTAGAAC GTTTGTCAAAG ATTCTCTAT GGACAGGTA GATATTTAAG CCCATGACTG
 -628 AGCTGGCTAC CACCCTGACC TATTTCTCC TGCTTCTCC AGAGCTGATG AAATCATTTGA CCCTCAGACT
 -558 CGTTTCTCT GGACCAAACCT GGACCATTA CGTGCTTCC CCCACAGCCC ACCCCCATTT ATCCTAGATA
 -488 CGATATCTAT CCTCCACCTC CCAGATGCAC AGGATATGCA CCCCAGGAGGA AGGGGAAATA AAACACTGCGCA
 -418 GACAAGGCCA GGATTCCTCC CCGCAGGTG AAGGGTGGG CGAAAAACATT GAGTGAGCCA CCCTTCCGGT
 -348 GCTCTTACT CAGGCGAGTC CTCTGTACTG TCTGAGGAG GAGAAGAAGCACGCTCTCAC CGACCCGCC
 -278 CCACATCCT GCACACACAGGGACTTGT AAGGAGGTGT GAGGCAGGGTGAGGACCCG CGTACAGCAG
 -208 TCAGGTCTTG TGAACACGC CGCATACAG TCCGCATCAT ACGTCACAG TGCTTCAACA GACCTGGGG
 -138 CTGGGCCCG GAGGAGCGGAGT CGCGTGG ACGAGGTGG CGCGGGCCTT CGCGGACCGA CCAACGGCGG
 -68 AGGCGAAGGGCGGAGCTAG CGGCGGGTA ACGGTAGCAA TAAAAGGCCG CGCGGGCGGG GAGGGCCAG
 ACGCATTTCT CTATACGCG AGAGCGGCTC GGCCGCGCG ACGCCGGTA TCCAGGCCGA GCCGGGAACG
 TCGAGAGCATG

Fig. 1. Nucleotide sequence of the 5' flanking region of the rat LAT-1 (L-amino acid transporter-1) gene (GenBank accession No. AF329652). The transcription initiation site, an A residue, is underlined at the +1 position. Putative *cis*-acting elements predicted by database analysis (MatInspector, TRANSFAC) are underlined. *AARE, amino acid response element consensus sequence 5'-(A/G)TT(G/T)CATCA-3' [13,17,18].

rat genomic library (GenomeWalker kit, Clontech) using a gene specific reverse primer 5'-CATCATAGTGGGCAC-CATCATCG-3'. Sequencing of this 2 kb region revealed an overlap (100% identity) with the 64 bp portion of 5' UTR in the rat LAT-1 gene sequence from GenBank (accession No. AB015432). Features of this novel sequence (GenBank accession No. AF329652), including putative *cis*-acting elements predicted by database analysis (MatInspector and TRANSFAC), are presented in Fig. 1.

To identify the transcription start site in the 5' flanking region of rat LAT-1 gene, we used both inverse RACE (rapid amplification of cDNA ends) (Fig. 2A) and primer extension (Fig. 2B) methods. The inverse RACE method is a modified RACE protocol described by Eyal et al. [19]. Using this method and the reverse primer 5'-CATCATAGTGGGCACCATCATCG-3' (Gibco), cDNA was synthesized from mRNA of the normal rat hepatic cell line WB [20] grown in the absence of arginine for 8 h. Following 5' phosphorylation and single-strand ligation of cDNA ends, the circular cDNA was used as a template for PCR using forward and reverse nested primers (for-

ward primers: 5'-AGGGCGAAGGCGTGACCCTG-CAGC-3' for lane 6 and 5'-AGGCGCGGGAGAAGAT-GCTGG-3' for lane 5 in Fig. 2A; reverse primer: 5'-ATGCTCTCGACGTTCCCG-3' for lanes 5 and 6 in Fig. 2A). The products (lanes 5 and 6 in Fig. 2A) were sequenced and confirmed to be derived from the TA1/LAT-1 gene. Based on analysis of the PCR product size and sequencing analysis, the transcription initiation site corresponds to an A residue 80 bp upstream from the translation start site.

Using primer extension to identify the transcriptional start site, the reverse primer 5'-CATCATAGTGGGCAC-CATCATCG-3' was end-labeled (using [γ - 32 P]ATP and T4 Polynucleotide Kinase, Promega). This labeled primer was then used to reverse transcribe mRNA from WB cells using Thermoscript (Gibco). The synthesized, radiolabeled, cDNA was analyzed on a urea/polyacrylamide gel according to the procedure described by Promega. The results, shown in Fig. 2B lanes 1 and 2, indicate a 240 bp fragment which corresponds to a calculated transcriptional start site at 80 bp upstream from the translation

start site. Thus, the transcription initiation sites defined by primer extension and inverse RACE methods are identical.

Numerous potential *cis*-acting elements were predicted by database analysis of the 2 kb long 5' flanking region (MatInspector and TRANSFAC, <http://dot.imgen.bcm.tmc.edu:9331/seq-search/gene-search.html>). These (shown in Fig. 1) include binding sites for Sp1, AP1 (activator protein 1), GR (glucocorticoid receptor), and myc/max. Several potential binding sites for repressor proteins were also predicted, such as the sites for δ EF1 and Gfi-1. Additionally, we found several sites which have 78–100% identity with the AARE (amino acid response element) consensus sequence [13,17,18]. It is currently unclear which of these putative elements may play a role in transcriptional regulation of TA1/LAT-1 under normal or pathological conditions.

For functional studies, promoter constructs containing progressive 5' deletion were made by PCR. The same reverse primer 5'-TTCCCGGCTCGGCCTGGATACC-3' was used for all constructs and various forward primers

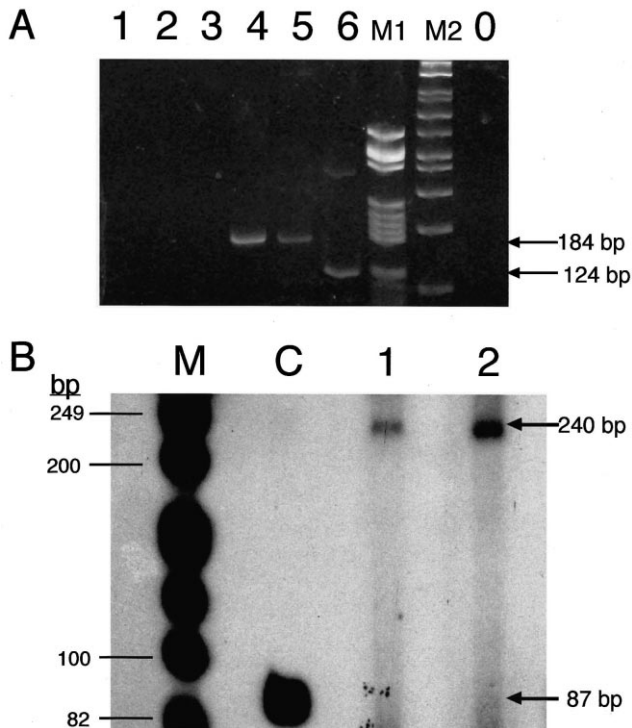


Fig. 2. Prediction of transcription initiation site by inverse RACE analysis (A) and primer extension method (B). In panel A, lane M1 is pBR322/*Hae*III marker (Sigma) and M2 is 1 kb Plus marker (Gibco). Lane 0 is the negative control for PCR with no template and lanes 1–3 are PCR products from reactions using single primer (each one of the reverse or forward primers as described in the text). Lane 4 is an internal positive control, amplified from the 180 bp LAT-1 coding region. Lanes 5 and 6 are products from WB hepatoma mRNAs using forward and reverse primers as described in the text. In panel B, lane M is the radiolabeled ϕ X174/*Hinf*I marker (Promega) and lane C is the positive control from Promega primer extension kit (kanamycin positive control RNA, 87 bp). Lanes 1 and 2 are primer extension products (240 bp) using radiolabeled primer as described in the text from WB hepatoma cell mRNAs (10 μ g and 50 μ g for lanes 1 and 2, respectively).

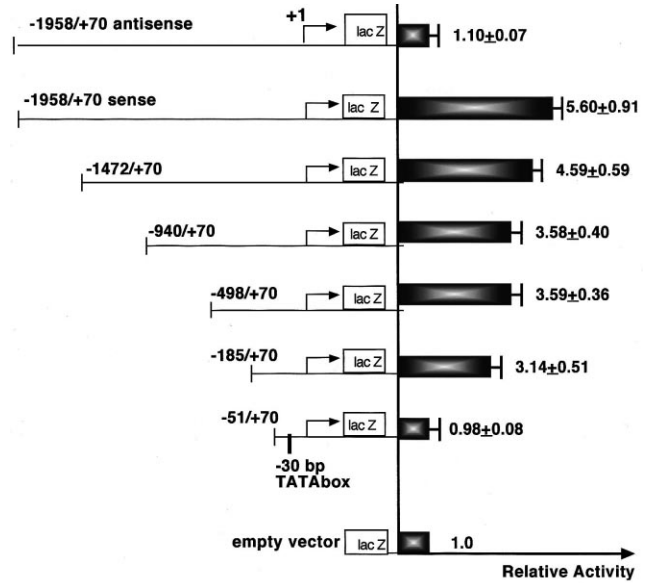


Fig. 3. Promoter constructs containing progressive 5' deletion and the corresponding relative activities in α mouse liver (α ML) cells. Relative activity (\pm S.E.M. of 6–10 data points from at least three independent experiments) is shown as fold activity of each construct relative to the activity of empty vector-transfected cell lysates. All activities were normalized to transfection efficiency as measured by the luciferase activity (PharMingen) of pGL3-CTL (Promega).

were used as follows: 5'-AATTCCCTGGCACCG-GACCTCC-3' for the $-1958/+70$ construct, 5'-TTTAG-CCCTCCGGAAGGACC-3' for the $-1472/+70$ construct, 5'-AGAGAGTCTTGGGGCTTCCTGG-3' for the $-940/+70$ construct, 5'-GATACAGTCCGCATCATAACG-3' for the $-185/+70$ construct, and 5'-TAGCGGGCGGGT-AACGGTAGC-3' for the $-51/+70$ construct. The construct $-498/+70$ was generated by PCR from the rat genomic library (GenomeWalker kit, Clontech), using adaptor primer supplied. Schematics of the constructs are illustrated in Fig. 3. These constructs were subcloned into a promoterless pBLUE-TOPO reporter vector containing a downstream *lacZ* gene (Invitrogen). An active promoter inserted into pBLUE-TOPO vector drives the *lac* gene encoding β -galactosidase enzyme whose activity can be monitored spectrophotometrically (β -galactosidase kit, Invitrogen).

All six promoter-reporter constructs as well as an empty vector (ligated promoterless reporter vector, Fig. 3) were transfected into the normal mouse hepatic cell line AML ($5\text{--}6 \times 10^6$ cells per 100 mm dish) using lipofectamine plus (Gibco). To normalize for transfection efficiency, the pGL3-CTL luciferase vector (Promega) under control of a strong eukaryotic promoter was co-transfected with each construct. Transfection was carried out in serum-free, antibiotic-free DMEM/F12 medium for 3 h. Following transfection, cells were grown in DMEM/F12/10% FBS. At 30–45 h post transfection, cells were harvested and cell lysates assayed for β -galactosidase activity (Invitrogen), luciferase activity (PharMingen), and protein concentrations (BCA

assay, Pharmingen). Results of these reporter assays (activities normalized to transfection efficiency) are summarized in Fig. 3. An average of 3.1–5.6-fold activities over control (empty vector) were seen for the constructs –185/+70, –498/+70, –940/+70, –1472/+70, and –1958/+70. The shortest construct (–51/+70) showed a low 0.98-fold activity, indicating that indispensable transcriptional activator site(s) are present in the region from –52 to –185. The longest construct (–1958/+70) showed the highest activity (5.6-fold), in contrast to the low 1.1-fold activity shown by the antisense version of this construct. Although the database analysis indicated putative elements for activators and repressors in these regions (see Fig. 1), further studies are needed to establish the interactions between these elements and the relevant factors.

In summary, we have cloned a 2 kb fragment of the 5' flanking sequence of the rat LAT-1 gene. To our knowledge, this is the first report on the cloning of an L-amino acid transporter gene promoter in any organism. The transcription initiation site was defined at 80 bp upstream from the translation start site by methods of inverse RACE and primer extension. Functional reporter analysis revealed regions which may be indispensable (–52 to –185) to transcriptional activity. This initial analysis permits future detailed examination of the basis for developmental and oncogenic regulation of TA1/LAT-1/CD98 light chain expression and, importantly, for response to arginine availability observed in non-transformed hepatic cells [12]. We are interested in the *cis*-elements and *trans*-acting factors which may be involved either directly or indirectly in LAT-1 transcription and its responsiveness to arginine. In this context, it is worth noting that sequence analysis predicted the presence of binding sites for factors such as aryl hydrocarbon receptor, which can be activated by amino acid [21], and upstream stimulatory factor (USF), which is involved in nutrient responses of certain liver genes [22]. It is also of interest to note that we found several sites which have 78–100% identity with the amino acid response element (AARE) consensus sequence [13], which was identified as an essential element in the regulated expression of the amino acid responsive genes asparagine synthetase [17] and human CHOP [18]. These studies are expected to further our general understanding of tumor-associated gene responses to the nutrient environment and may have important implications in dietary

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