LOT1 (PLAGL1/ZAC1), the Candidate Tumor Suppressor Gene at Chromosome 6q24–25, Is Epigenetically Regulated in Cancer*

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LOT1 is a zinc-finger nuclear transcription factor, which possesses anti-proliferative effects and is frequently silenced in ovarian and breast cancer cells. The LOT1 gene is localized at chromosome 6q24–25, a chromosomal region maternally imprinted and linked to growth retardation in several organs and progression of disease states such as transient neonatal diabetes mellitus. Toward understanding the molecular mechanism underlying the loss of LOT1 expression in cancer, we have characterized the genomic structure and analyzed its epigenetic regulation. Genome mapping of LOT1 in comparison with the other splice variants, namely ZAC1 and PLAG1, revealed that its mRNA (~4.7 kb; GenBank accession number U72821) is potentially spliced using six exons spanning at least 70 kb of the human genome. 5′RACE (rapid amplification of cDNA ends) data indicated the presence of at least two transcription start sites. We found that in vitro methylation of the LOT1 promoter causes a significant loss in its ability to drive luciferase transcription. To determine the nature of in vivo methylation of LOT1, we used bisulfite-sequence strategies on genomic DNA. We show that in the ovarian and breast cancer cell lines and/or tumors the 5′-CpG island of LOT1 is a differentially methylated region. In these cell lines the ratio of methylated to unmethylated CpG dinucleotides in this region ranged from 31 to 99% and the ovarian tumors have relatively higher cytosine methylation than normal tissues. Furthermore, we show that trichostatin A, a specific inhibitor of histone deacetylase, relieves transcriptional silencing of LOT1 mRNA in malignantly transformed cells. It appears that, unlike DNA methylation, histone deacetylation does not target the promoter, and rather it is indirect and may be elicited by a mechanism upstream of the LOT1 regulatory pathway. Taken together, the data suggest that expression of LOT1 is under the control of two epigenetic modifications and that, in the absence of loss of heterozygosity, the biallelic (two-hit) or maximal silencing of LOT1 requires both processes.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U72621. To whom correspondence should be addressed: Ovarian Cancer Program, Dept. of Medical Oncology, Fox Chase Cancer Center, 7101 Burholme Ave., Philadelphia, PA 19111. Tel.: 215-728-3679; Fax: 215-728-2741; E-mail: A_Abdollahi@fccc.edu.

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LOT1 (lost-on-transformation 1) is a growth suppressor gene (1, 2) localized on chromosome 6 at band q24–25, which is a frequent site for loss of heterozygosity in many solid tumors including ovarian cancer (2, 3). The mouse ortholog of this gene was independently identified by Spengler et al. (4) and designated as Zac1, which is highly homologous to the hLOT1 and rLOT1 genes. A splice variant of LOT1/ZAC1 was identified by Kas et al. (5) and named as PLAG1 based on its homology with PLAG1 protein encoded by the gene PLAG1 localized on chromosome 8q12 (6). The presence of other genes with high homology to LOT1/ZAC1/PLAG1 indicates that this gene is a member of a new family of zinc-finger proteins. Our studies and those of others have shown that LOT1 gene expression is frequently down-regulated in the ovarian and breast carcinoma cells (1, 2, 7). Functional analysis of LOT1 demonstrated that it may play a significant role as a transcription factor modulating growth suppression through mitogenic signaling pathways (8).

It is increasingly evident that epigenetic modification of genomic DNA by methylation and/or histone deacetylation plays an important role in transcriptional silencing and loss of gene function of certain tumor suppressor genes. Methylation of DNA normally occurs at cytosine residues within CpG dinucleotides in almost all higher eukaryotic organisms (9–11). This type of modification can suppress gene expression directly by interfering with the binding of transcription factors or indirectly by initiating a complex consisting of methyl-CpG-binding proteins (e.g. MeCP2, MBDs) and histone deacetylases (12–16). These complexes mediate transcriptional repression through chromatin hypoacetylation, which can be partially reversed by TSA. In human cancers, aberrant methylation of CpG islands silencing the promoters of some genes that function in the suppression of malignant phenotype has been found. For example hypermethylation of CpG islands in different cancer cells has been implicated in the transcriptional inactivation of the Rb, p16, estrogen receptor, INK4A/MTS2, VHL, and H19 genes (17–24). Similarly, CpG island methylation may play a significant role in the regulation of imprinted genes (25) and genes located on the inactive X chromosome (26, 27).

Recently, the LOT1/ZAC1 locus at chromosome 6q24–25 was identified as a maternally imprinted region (2, 4, 28–30). These studies have established that the paternal duplication or loss of maternal imprinting of the LOT1 locus is associated with transient neonatal diabetes mellitus, an imprinted disease charac-

* The abbreviations used are: LOT1, lost-on-transformation 1; TSA, trichostatin A; ROSE, rat ovarian surface epithelial; RACE, rapid amplification of cDNA ends; UTR, untranslated region; FACS, fluorescence-activated cell sorter; GFP, green fluorescence protein; PBS, phosphate-buffered saline; ChIP, chromatin immunoprecipitation; TSS, transcription start site; ORF, open reading frame; HDAC, histone deacetylase.
In this study, we have characterized the LOT1 gene structure with relation to different splice variants and identified the gene’s minimal promoter and a CpG island. Our results indicate that LOT1 is subject to two epigenetic processes, methylation of CpG islands and histone deacetylation, which may synergistically act to regulate the transcriptional silencing of the gene.

MATERIALS AND METHODS

Cells and Cell Cultures—The ovarian cells were maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum, glutamine (2 mM), insulin (10 μg/ml), penicillin (100 units/ml), and streptomycin (100 μg/ml).

Cloning of Full-length LOT1 and 5′-RACE—To obtain additional sequence information of the 5′-untranslated region (5′-UTR) of hLOT1 cDNA, which we previously reported (2), a normal human fetal brain cDNA library in AZAPII vector (Stratagene, La Jolla, CA) was used. A positive clone, FB2, which hybridized to the hLOT1 probe, gave extended 5′-UTR sequences. Insert sequences of all plasmids were determined by automated ABI PRISM dye terminator cycle sequencing with FS AmpliTaq DNA polymerase and run through an Applied Biosystem 373/377 sequencer (ABI/PerkinElmer Life Sciences). The data were analyzed using FASTA and PILEUP DNA sequence homology searches in the Wisconsin Genetics Computer Group package (Madison, WI).

We predicted the human LOT1 promoter, a human placental genomic DNA library (EMBL-3-Sp6/T7, Clontech, Palo Alto, CA) was screened using the following fragments in the hLOT1 cDNA (GenBank accession number U72621.3) as probes: a fragment containing the complete open reading frame; b) a 271-bp fragment upstream of the LOT1 translation start codon. This plasmid was purified with the lysis buffer, and centrifuged, and 5′ ends of the RNA were ligated into pG3-Basic reporter vector (Promega) containing the luciferase gene, which was previously digested with either XhoI alone (pG3-60) or XhoI and HindIII (pG3-45, pG3-47) restriction enzymes. The ligation reactions were used to transform DH5a Escherichia coli competent cells and the clones were confirmed by sequencing their insert. All plasmids used for the transfections were purified using the Qiagen kit.

In Vitro DNA Methylation—The promoter construct pG3-45 was in vitro methylated by the bacterial methylase, SsI (New England Biolabs), or mock-treated in the absence of enzyme. The DNA was incubated for 24 h at 37 °C with SsI methylase at 1 unit/μg DNA in 50 mM sodium acetate, 10 mM MgCl2, and 1 mM dithiothreitol (pH 7.9) supplemented with 160 μM S-adenosylmethionine.

Construction of GFP Fusion Proteins, Fluorescence Microscopy, and FACS Analysis—GFP-tagged LOT1 mammalian expression vector was constructed as described previously (8). Briefly, the GFP coding region was amplified using forward (5′-CCCATGGCTAGCAAGGGCCAG-3′) and reverse (5′-TTCTGATACGGCTCCTGACG-3′) primers and reverse (5′-TTCTGATACGGCTCCTGACG-3′) primers. The PCR product was ligated with XhoI linker to the LOT1 coding region, and the resulting fragment (GFL) was introduced downstream of the cytomegalovirus promoter into the XhoI site of pcDNA3 plasmid. A2780 cells were cultured in RPMI 1 day before transfection. Transient transfections were performed using TransIT-LT1 transfection reagent (PanVera, Madison, WI) according to the manufacturer’s instructions. Cells (2 × 105/well) were seeded in 6-well plates and transfected with 4 μg/pL of pcDNA/GFP vector carrying GFP-LOT1 fusion protein. The cells were then monitored 18 h later and imaged with a Quantix 12-bit cooled charge-coupled device camera (Roper, Inc., Tuscon, AZ). Fluorescent GFP and phase contrast images were generated simultaneously using Isee software (Invision Corp., Durham, NC) to charge-coupled device, a Luidd filter wheel, and a shutter attached to a Nikon TE300 inverted microscope. The cells were observed at near-physiological conditions using a forced-air incubator, which encompasses the microscope. For FACS analysis the transfected cells were collected by trypsinization, washed, and resuspended in PBS. The cell suspensions were analyzed using a flow cytometer (Becton Dickinson FACSCals VintageSE flow cytometer (Becton Dickinson, Inc., San Jose, CA)). 10,000 cells were subjected to FACS analysis.

Transient Transfection and Luciferase Assay—Ovarian cancer cells at a density of 4 × 105/well were cultured in 6-well plates in RPMI 1640 medium 1 day prior to transfection with the reporter plasmid as indicated. Transient transfections were performed using TransIT-LT1 polyamine transfection reagent (PanVera, Madison, WI) according to the manufacturer’s instructions. Briefly, the cells were washed once with serum-free medium and incubated with the DNA (2 μL/6 μL) reagent mixture for 5 h in serum-free medium followed by additional incubation for 17 h (overnight) in fresh medium containing 10% fetal bovine serum. The cells were then washed using the Dual Luciferase Assay System (Promega). The cells were washed with 1× PBS, harvested with the lysis buffer, and centrifuged, and 5–20 μL of the cell lysate (supernatant) was added to 100 μL of the luciferase substrate. The luciferase enzyme activity was measured immediately or within 15 s of adding the substrate, luciferin, using the Monolight (R) 2010 Luminometer (Analytical Luminescence Laboratory, Inc.). This data was analyzed using the Dual-Luciferase Assay System (Promega).

Northern and Western Blot Analysis—Total RNA was isolated from the cells by the guanidinium isothiocyanate extraction method (35) or Trizol (Invitrogen) and was separated on 1% agarose gels containing 2.2 M formaldehyde. The RNA was transferred to Nylon membranes (Micron Separations, Inc.) by capillary action and hybridized using a probe specified in the legend. Northern and Western blot hybridizations were carried out using a kit specified in the legend.

Construction of Luciferase Expression Vectors—The genomic structure of LOT1 was characterized by intrauterine fetal growth retardation and insulin dependence. However, little is known about the molecular mechanisms that down-regulate expression of LOT1 in cancer.
incubation with the transfection reagent supplemented with the appropriate DNAs, whole-cell lysates were prepared using 250 μl/well of M-PER Protein Extraction Reagent (PIERCE, Rockford, IL) containing phenylmethylsulfonyl fluoride and diithiothreitol. Equal volumes of protein (15 μl) from each sample were electrophoresed on SDS-PAGE (10%) followed by transfer to a Hybond nylon membrane (Amersham Life Science, UK) for Western blot analysis. Immunoblots were blocked overnight in 5% nonfat dry milk (w/v) in TBST containing 20 μM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween 20. Blots were incubated for 1 h with anti-GFP monoclonal antibody (Clontech, Palo Alto, CA) diluted with 1% nonfat dry milk in TBST. The blots were washed with TBST three times, and incubated for 1 h with the secondary antibody (anti-mouse IgG horseradish peroxidase, Amersham Biosciences). The DNA was digested overnight at 37°C with restriction digest-PCR (PerkinElmer Life Sciences) and exposure to x-ray films (Kodak Co, Rochester, NY).

DNA Isolation, Bisulphite Sequencing, and Methylation-sensitive Restriction Digest-PCR—Genomic DNA was isolated by digestion with proteinase K (Invitrogen) followed by phenol/chloroform extraction (36). After precipitation by the addition of 2 volumes of ethanol and 0.5 volumes of ammonium acetate (7.5 m), the DNA was washed twice with ethanol (80%, v/v), and dissolved in water. Normal and tumor DNA samples were obtained from Biosample Repository Core Facility (Andrew Klein-Szanto and Robert Page) at Fox Chase Cancer Center. Genomic DNA (1 μg) was treated with sodium bisulphite and used for methylation-sensitive PCR as previously described (37, 38). Briefly, the DNA in a volume of 50 μl was denatured by 0.2 μl NaOH and then treated with hydroquinone (Sigma) and sodium bisulphite (Sigma) at 50°C for 16 h. Bisulphite-modified DNA was purified using Wizard DNA Clean-Up system (Promega). Modification was completed by NaOH (0.3 m) for 5 min at room temperature.

The bisulphite-treated DNA was precipitated by glycogen, ammonium acetate, and 3 volumes of ethanol, and the pellets were washed once with 70% ethanol and dissolved in 20 μl of water. The modified DNA (4 μl) was used as PCR under the following reaction conditions: 2 μl of 10× Taq buffer, 125 μM dNTPs, 7.5 mM MgCl2, and 1 μM of each primer in a 50-μl reaction. The reaction was carried out with the cycling condition of denaturation at 95°C for 10 min followed by the addition of 0.5 unit of Gold Taq polymerase (Invitrogen) and 32 cycles of PCR (95°C, 30 s; 58°C, 30 s; 72°C, 30 s) with a final extension of 4 min at 72°C. Second round of PCR was performed using 2 μl of the first PCR and the same reagents and conditions as above. Primers were designed from the interologated sequence after bisulphite conversion assuming DNA was either methylated or unmethylated at 14 CpG sites or the imprinting interpolated sequence after bisulfite conversion assuming DNA was for screening, and a positive clone, FB2, hybridized to the hLO1 probe and produced an extension of the 5′-UTR sequence. This newly identified nucleotide sequence of the LOT1 gene was added to the previously published sequence that is stored in GenBankTM with the accession number U72621.

We identified the transcription start site (TSS) of LOT1 using 5′-RACE technique. The cDNA for 5′-RACE was derived from human ovary and generated based on oligo-capping and RNA ligase-mediated RACE methods (33, 34) as described in the GeneRacer cDNA Kit (Invitrogen). We detected a strong PCR band corresponding to about 390 nucleotides in size and weaker bands that are smaller or larger (Fig. 1). Two of the smaller bands are shown with arrows (about 280 and 200 bp). The RACE product was cloned and sequenced. Nine of 35 clones contained the LOT1 sequence, and the others were unrelated. Analysis of the amplified 5′ ends predicted two potential transcription start sites, TSS1 and TSS2, as are shown in Fig. 1. Two of the positive clones had the 5′ end at nucleotide 53080 (TSS1) and six clones started at nucleotide 52919 (TSS2) of clone RP3–340H11 on chromosome 6q (GenBank accession number AL109755), resulting in an alternative exon of exon 1 (326 and 165 nucleotides, respectively). Interestingly, an expressed sequence tag clone (accession number B1515851) constructed using the Cap-trapper method also showed the 5′ end at TSS2. These data suggest that the LOT1 message is preferentially transcribed from these start sites, at least in the ovary.

Analysis of the RACE-PCR sequences also revealed that all the positive clones (100%) were extended 5′ upstream from the gene-specific reverse primer into the LOT1 splice variant at the junction of exons 1 and 2, as opposed to the ZAC1 splice variant, which diverges from LOT1 at the 5′ end of exon 2 in LOT1 (exon 3, ZAC1) (Fig. 1) (see below). In addition, sizes of the bands on the gel (Fig. 1), as shown by arrows, correspond to the fragments (234 and 396 bp) from the LOT1 splice variant at this 5′ upstream region. Taken together, the sequence analysis and hybridization results using different regions of hLO1 or rLO1 as probes (data not shown) suggest that human LOT1 (accession number U72621) and rat LOT1 (accession number U72620) represent the major transcripts for this gene in the human and rat, respectively (1, 2, 8).

Analysis of the LOT1 Splice Variants—To obtain the genomic structure of hLO1 we first began analyzing the open reading frame (ORF). Several sets of primers were designed in the
coding region and used to amplify normal genomic DNA. The primers p63 and p69 generated a PCR product that encompassed the majority of the ORF. The sequence data did not reveal any evidence of introns from zinc-finger four to the 3’ end of the ORF (data not shown). Screening a placental DNA library indicated that the sequence between the intron/exon boundaries of the LOT1 gene (Fig. 2), which does not display a TATA box element. The LOT1 peptide is encoded by exons 5 and 6, resulting in a sequence at this diverging point. The RACE clones for LOT1 and PLAGL1 (5, 40–42) and subsequently with the genomic clones in the GenBank (AL109755 and AL049844). The data are shown in Fig. 3, which depicts the relative location and size of exons. These variants have a common 3’ end on exon 1; however, the subsequent exons are differentially spliced, resulting in six, nine, and five exons for LOT1, ZAC1, and PLAGL1, respectively (Fig. 3). Interestingly, LOT1 and ZAC1 variants have an identical open reading frame. However, the open reading frame of PLAGL1 lacks all the amino acid sequences upstream of the second methionine in the LOT1 and ZAC1 sequence (5).

Characterization of the Human LOT1 Promoter—We then cloned the 5’ flanking segments of the LOT1 gene into the pGL3-Basic vector (Promega, Madison, WI) upstream of the luciferase gene to determine whether this region possesses transcription activity. The structures of the different constructs are schematically shown in Fig. 4A, and the results of a representative experiment from at least two similar experiments using these plasmids are presented in Fig. 4B. As is shown in Fig. 4B(a), insertion of the −1.5-kb human LOT1 genomic fragment (nucleotides −1456 to −1 with respect to the 5’ end of LOT1 sequence in GenBank accession number U72621) into pGL3, designated as pGL3−45, resulted in a strong increase (up to −35-fold) in the activation of the luciferase gene in the A2780 ovarian cancer cell line. The transcription activity was abolished following the 5’ truncation of the promoter, as is shown for pGL3−47 (nucleotides −525 to −1) plasmid. Interestingly, however, a second but weaker promoter activity (−10-fold compared with −35-fold for pGL3−45) was observed with the exon 1 proximal region (nucleotides −123 to +450; vector pGL3−60) as is apparent from Fig. 4B(b), suggesting the presence of at least two potentially active promoter regions in the LOT1 gene.

We also confirmed the promoter activity in other cell lines including the human ovarian cell line OVCAR3 and the ROSE cell line NuTu 26. Transfection of these cells with the expression vectors pGL3−45 and pGL3−60 showed a considerable level of reporter activity (Fig. 4C), although the transcription activity was lower in the ROSE cells than OVCAR3 cells. Activation of the human LOT1 promoter in ROSE cells was expected since we found the hLOT1 promoter sequence to be highly homologous to the mouse Lot1 promoter (and therefore likely to the rat Lot1 promoter) by aligning different segments of the hLOT1 promoter with that of the mouse.

![Fig. 2. Genomic structure of LOT1. A, the six exons in LOT1 and the introns are shown (q = long arm of chromosome 6; TEL = telomere; CEN = centromere). B, exon/intron boundaries were determined by subcloning different fragments obtained from a phage DNA, P1 genomic clone, and total genomic DNA using PCR with LOT1-specific primers or restriction digest analysis. The nucleotide sequences of these subclones were aligned to the LOT1 cDNA sequence (GenBank accession number U72621). The numbers on each intron indicate the approximate size of nucleotide sequence.](http://www.jbc.org/content/264/16/6044/F2.large.jpg)
could be reactivated. The results showed that in these cell lines cytidine to determine whether the expression of LOT1/ZAC1 cancer cell lines with the demethylating agent 5-aza-2/H11032. Furthermore, we treated MDA-MB-453, CAOV3, and NuTu 26 expression data and the methylation status in these cell lines. We have previously shown that the ovarian cancer cell lines express differential levels of data. We were not able to find a direct association between the data. It is increasingly evident that methylation of CpG-island that spans the nucleotides 54333–52440 on chromosome 6q (GenBank accession number AL109755) (Fig. 5). The sequences between nucleotides 53160 and 52440 or the region on exon 1 and part of the first intron have the highest content of CpG dinucleotides (Figs. 1 and 5). Therefore, we examined the methylation status of specific sites within this CpG island or the imprinting control region (28, 29) near the transcription start site (Fig. 5) by bisulfite-PCR-sequencing analysis. The genomic DNA isolated from cancer cell lines and tissues was bisulfite-treated, which converts unmethylated but not methylated cytosine into uracil, as is shown in Fig. 6. The bisulfite-treated DNA was subjected to PCR and subcloned into a TA-cloning vector, and independent clones were sequenced. Completion of the bisulfite reaction was judged by the efficient conversion of all the cytosine residues that do not precede guanines. We then determined the ratio of methylated sites versus unmethylated sites, and the data were recorded as percent. As shown in Fig. 7A, the ovarian cancer cell lines (seven cell lines) had between 31 and 52% methylation, and the cell line CAOV3 had 99% methylation of the CpG sites on the LOT1 locus. In the two breast cancer cell lines, MDA-MB-453 and -468, the methylation levels were 93 and 58% (Fig. 7B), respectively. Hence, in these ovarian and breast cancer cell lines the alleles containing one or more methylated CpG dinucleotides in the differentially methylated region ranged from 50 to 99%. It is worth mentioning that we also subjected the DNA from MDA-MB-453 cells to bisulfite/PCR/restriction enzyme (TaqI enzyme, BioLab) analysis and found that the DNA was hypermethylated, confirming the sequencing data. We have previously shown that the ovarian cancer cell lines express differential levels of LOT1 message (1). However, we were not able to find a direct association between the expression data and the methylation status in these cell lines. Furthermore, we treated MDA-MB-453, CAOV3, and NuTu 26 cancer cell lines with the demethylation agent 5-aza-2′ deoxycytidine to determine whether the expression of LOT1/ZAC1 could be reactivated. The results showed that in these cell lines LOT1 is unresponsive to 5-aza-2′ deoxycytidine treatment (data not shown). These results suggest that additional mechanisms other than methylation may be involved in the regulation of LOT1. In other words, we hypothesize that in some cases constitutive down-regulation of LOT1 may be due to alteration elsewhere upstream in the pathway and that direct demethylation of CpG dinucleotides may not be sufficient to restore the normal expression pattern of this gene.

We examined the methylation status in paired cancerous and non-cancerous ovarian tissues and found that in three sample pairs (59, 151, 001) the tumor DNA had higher methylation level than in the normal DNA and in another pair (1002582) the difference was insignificant (Fig. 7C). We also examined the methylation status of normal ovary DNA with methylation-sensitive restriction enzyme-PCR. The DNA was digested with HpaII or MspI. These restriction enzymes both cleave at CCGG sites. However, HpaII does not cut the DNA when the internal C is methylated, and MspI digests the DNA regardless of methylation status. The DNA was then subjected...
Recent studies have revealed that histone deacetylase inhibition failed to relieve methylation-mediated repression of transcription from this promoter fragment (data not shown). In addition, we performed ChIP assay of cross-linked chromatin isolated from the control and TSA-treated A2780 (non-transfected) cells with antibodies against acetylated H4 histone and PCR amplified the LOT1 promoter. The data as shown in Fig. 9A indicate that the association of acetylated histone with the promoter is not significantly changed in response to TSA treatment. Therefore, we argued that the TSA treatment may target the LOT1 message due to an indirect effect originated upstream of the LOT1 gene. To test this hypothesis, we fused the LOT1 cDNA downstream of the green fluorescent gene and used the construct to transfet A2780 ovarian cancer cells. The data presented in Fig. 9B show that the number of positive cells expressing GFP-LOT1 fusion protein is amplified following the inhibition of HDAC, as determined by fluorescence image analysis. Similar results were obtained with the NuTu 26 cell line, which has lost or decreased expression of the gene (Fig. 8) (data not shown). Western blot analysis indicated that the GFP-LOT1 protein level, compared with GFP alone, is significantly increased in the cells treated with TSA (Fig. 9C).

The effect of inhibition of histone deacetylase observed in Fig. 9, B and C was also confirmed by FACS analysis and cell sorting of the GFP-expressing cells. As is shown in Fig. 10, the number of positive GFP-LOT1-expressing cells was lower than that of GFP-expressing cells and that the number increased significantly upon the stimulation with TSA. Taken together, these data and the Northern analysis (above) show clear correlation between the transcriptional responses of the LOT1 gene to TSA, exogenously and endogenously.

**DISCUSSION**

The candidate tumor suppressor gene LOT1 encodes a nuclear transcription factor and is strongly regulated by the activation of the epidermal growth factor receptor signaling pathway (1, 2, 8). In this paper, we have reported cloning of the
full-length LOT1 cDNA, characterized the genomic structure, and analyzed its epigenetic regulation. We have also determined the exon/intron splice junctions of LOT1 and its splice variants, namely ZAC1 and PLAGL1. The LOT1 gene consists of six exons and spans at least 70 kb. The introns range from about 2.6 to 39 kb in size. The LOT1 peptide is encoded by exons 5 and 6, resulting in a large 5'UTR. A CpG-rich island is present in exon 1 and extends into the 5' flanking region of the gene, which does not display a TATA box element. We predicted the TSS of LOT1 using the 5'-RACE technique. Analysis of the amplified 5'-ends using cDNA obtained from whole normal ovary predicted two potential transcription start sites, TSS1 and TSS2. However, the absence of a TATA box within the 5'-upstream sequences of LOT1 suggests that the actual TSS of transcription may be heterogeneous. We have identified a putative promoter region within 1.5 kb 5'-upstream of LOT1. Insertion of the ~1.5-kb fragment into pGL3 resulted in a strong induction (up to ~35-fold) of the luciferase gene in the ovarian cancer cell lines. Interestingly, however, a second but weaker promoter activity (~10-fold) was observed with the exon 1 proximal region (Fig. 5), suggesting the presence of at least two potentially active promoter region in the LOT1 gene.

We previously hypothesized that the LOT1 gene expression may be regulated by mechanism(s) that may involve histone acetylation/deacetylation, DNA methylation, and/or mRNA stability (8). We have identified CpG islands in the upstream sequences of exon 1 and into the promoter of LOT1, which suggest that DNA methylation of CpG nucleotides may play a role in silencing the gene expression. In addition, recent reports suggested that LOT1/ZAC1 is part of a maternally imprinted chromosomal region (28–30). Therefore, we have analyzed the imprinting based on methylation of the CpG-region and found that DNA from both normal ovary and ovarian cancer cell lines exhibit methylation of the cytosine residues. It appears that ovarian tumors and cancer cells demonstrate relatively increased levels of methylation in CpG dinucleotides. The breast cancer cell lines also had high levels of methylation. The presence of methylated alleles in normal ovary DNA suggests that imprinting of the maternal allele of the actual LOT1 gene is maintained in this tissue, and possibly in breast or other somatic cells, during mammalian development. Thus, based on these results, it is rational to believe that the LOT1 gene may be subject to two-hit inactivation or silencing by loss of heterozygosity and methylation.

Furthermore, in this study we have presented evidence for the possible role of histone deacetylation in repressing the LOT1 gene expression. Treatment of the cells with trichostatin A, a potent inhibitor of histone deacetylase, reverses the gene's
silencing in NuTu 26 and A2780 ovarian cancer cell lines. These results suggest that HDAC activity plays a role, at least in part, in regulating the LOT1 gene. Direct interaction of HDACs with either the acetylated histones (H2A, H2B, H3, and H4) or non-histone proteins may result in deacetylation of these proteins and regulation of gene transcription. Deacetylation of histones has been found to stabilize the genomic DNA/histone complex, hindering accessibility of the promoter to the transcription machinery (43). Apparently, this process is further enhanced by DNA methylation, which may promote histone deacetylation and/or may assist in recruiting methylated DNA-binding protein MeCP2 to the regions of DNA by the HDACs/co-repressor complex (45–48). However, in this study we found that TSA treatment does not reactivate the in vitro methylated LOT1 promoter and does not significantly change the promoter DNA-acetylated histone association, suggesting that there exists a histone deacetylase-independent mechanism(s) for gene silencing of this locus by DNA methylation, as has also been described for other loci (49). Consistent with our findings, it has been recently reported that treatment of maternal uniparental mouse embryonic fibroblasts with TSA does not result in the inheritable expression of the imprinted Lot1/Zac1 gene (50). This notion is supported by other reports that in cancer the hypermethylated genes such as MLH1, TIMP3, CDKN2B/INK4B/p15, and CDKN2A/INK4/p16 can not be transcriptionally reactivated with TSA alone (51). Therefore, it is possible that re-expression of LOT1 by the inhibition of histone deacetylase may be due to the effect on the unmethylated or hypomethylated allele of the gene and be elicited by a process originating elsewhere upstream in the LOT1 pathway. We conclude that distinct mechanisms other than imprinting...
methylation may play major roles in regulating the LOT1 expression, particularly when the expression needs to be completely blocked, e.g. expression from the paternal allele or from the maternal allele when imprinting may be erased during development. Perhaps, promotion of cell survival and positive expression, particularly when the expression needs to be competed.

Studies are currently underway to elucidate in detail the mechanism of LOT1 gene regulation by different genetic and epigenetic factors, which may be useful in determining precisely the role of this gene in cancer and/or development.

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