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ZAC, *LIT1* (*KCNQ1OT1*) and *p57^{KIP2}* (*CDKN1C*) are in an imprinted gene network that may play a role in Beckwith–Wiedemann syndrome

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ABSTRACT

Loss of genomic imprinting is involved in a number of developmental abnormalities and cancers. *ZAC* is an imprinted gene expressed from the paternal allele of chromosome 6q24 within a region known to harbor a tumor suppressor gene for several types of neoplasia. *p57^{KIP2}* (*CDKN1C*) is a maternally expressed gene located on chromosome 11p15.5 which encodes a cyclin-dependent kinase inhibitor that may also act as a tumor suppressor gene. Mutations in *ZAC* and *p57^{KIP2}* have been implicated in transient neonatal diabetes mellitus (TNDB) and Beckwith–Wiedemann syndrome, respectively. Patients with these diseases share many characteristics. Here we show that mouse *Zac1* and *p57^{Kip2}* have a strikingly similar expression pattern. ZAC, a sequence-specific DNA-binding protein, binds within the CpG island of *LIT1 (KCNQ1OT1)*, a paternally expressed, anti-sense RNA thought to negatively regulate *p57^{KIP2}* in *cis*. ZAC induces *LIT1* transcription in a methylation-dependent manner. Our data suggest that *ZAC* may regulate *p57^{KIP2}* through *LIT1*, forming part of a novel signaling pathway regulating cell growth. Mutations in *ZAC* may, therefore, contribute to Beckwith–Wiedemann syndrome. Furthermore, we find changes in DNA methylation at the *LIT1* putative imprinting control region in two patients with TNDB.

INTRODUCTION

Genomic imprinting is a gamete-specific modification that results in the differential expression of the two parental alleles in somatic cells (1,2). Mutations that affect the epigenetic status of imprinted loci are involved in a number of human diseases, developmental abnormalities and malignant tumors indicating a general role for imprinted genes in mammalian development (3). Several imprinted genes, including *INS2*, *p57^{KIP2}* (*CDKN1C*), *GNAS*, *RASGRF1*, *MASH2* and *ZAC* play a role in regulating the cell cycle (4).

ZAC was originally identified, along with *p53*, in a functional screen for factors that induce expression of the pituitary adenylate cyclase activating polypeptide (*PACAP*) type I receptor gene (5). ZAC encodes a zinc finger protein and is expressed only from the paternal allele with the maternal allele silent and methylated (6–8). Mutations in *ZAC* are thought to play a role in transient neonatal diabetes mellitus (TNDM). The gene maps to 6q24–25, a region implicated in the origin of several cancers (9–12). Abdollahi *et al*. (13) reported a high incidence of allelic loss at this chromosomal region in ovarian cancers. In addition, they independently cloned as *Lot1* (Lost on transformation), the rat ortholog of *ZAC*, from rat ovarian surface epithelial cells that spontaneously transform *in vitro*.

ZAC exhibits a tumor suppressor activity characterized by induction of apoptosis and G1 arrest. This proceeds independently of known cell cycle control proteins, such as pRB, p21, p27 and p16 (5). Nevertheless, ZAC shares a number of similar functions to p53. Both proteins regulate the cell cycle, apoptosis and nuclear receptors. They interact physically and functionally with CBP and p300 that serve as integrators of multiple signaling pathways (14,15). Both p53 and ZAC are

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sequence-specific DNA-binding proteins and can act as transcriptional co-factors for other transcriptional activator proteins (16,17). Recently, ZAC was shown to specifically enhance the activity of p53-responsive promoters in cells expressing wild-type p53 (18). It is, therefore, possible that ZAC may act as a transcription factor with a similar mode of action to p53.

Three types of mutations have been shown to result in TNDM; paternal uniparental isodisomy of chromosome 6, paternally inherited duplication of 6q24 and a methylation defect at a CpG island overlapping exon 1 of *ZAC*/*HYMAI* (19). We showed that this CpG island is differentially methylated (20). The region is unmethylated in sperm but methylated in oocytes, a difference that persists between parental alleles throughout pre- and post implantation development suggestive of an imprint control region (ICR). There is a region within this putative ICR that exhibits a high degree of homology between mouse and human that acts as a strong transcriptional repressor when methylated. Loss of methylation at 8 CpG sites within this region was seen in five of six TNDM patients studied with a normal karyotype. We proposed that the DMR adjacent to *ZAC* may regulate expression of imprinted genes within the domain, and that epigenetic or genetic mutations of this region result in TNDM by affecting expression of *ZAC* in the pancreas and/or the pituitary (20). This is supported by the recent findings that mice overexpressing human ZAC from a transgene show impaired development of the endocrine pancreas and impaired β-cell function (21). ZAC may mediate its actions through transcriptional regulation of the *PACAP* type I receptor gene which is a potent insulin secretagogue and an important mediator of autocrine control of insulin secretion in the pancreatic islet (20).

During our studies on mouse *Zac1* (20), we noted a similarity in the expression pattern of *Zac1* with a second imprinted gene, $p57^{Kip2}$ (*Cdkn1c*), hereafter referred to as $p57^{Kip2}$. $p57^{KIP2}$ is a maternally expressed gene that encodes a cyclin-dependent kinase inhibitor. The protein inhibits all G_1 cyclin/Cdk complexes and negatively regulates cell proliferation, acting downstream of p53. Like *ZAC*, $p57^{KIP2}$ may also act as a tumor suppressor through its role in regulating the cell cycle (22–24). $p57^{KIP2}$ is located on human chromosome 11p15.5, a region implicated in both sporadic cancers and Beckwith–Wiedemann syndrome (BWS), a familial cancer syndrome, making it a candidate tumor suppressor gene (23). Several types of childhood tumor display a specific loss of the maternal 11p15 allele that suggests the involvement of an imprinted gene in this region (25).

BWS patients generally present with three major features: exomphalos, macroglossia and giantism. Other features include hypoglycemia, organomegaly, hemihypertrophy, genitourinary abnormalities, cleft palate and a susceptibility to embryonal tumors. In turn, TNDM is associated with intrauterine growth failure, dehydration, hyperglycemia and failure to thrive (26,27). There have been a number of reported associations with TNDM including umbilical hernia (7%) and macroglossia (23%) (28). These two anomalies are also found in patients with BWS, which typically also involves hyperinsulinemia. The role for *ZAC* is a predicted gain of function with biallelic expression of *ZAC* in TNDM patients (since UPD of the region also results in TNDM). Loss of function of $p57^{KIP2}$ in BWS is implicated in patients with point mutations within the coding sequence of the gene (29–32).

Several imprinted genes lie within the 11p15.5 region. *LIT1* (*KCNQ1OT1*), hereafter referred to as *LIT1*, is a paternally expressed, antisense RNA located within the *KCNQ1* gene that may regulate imprinting of the 11p15.5 domain (25). Differential methylation of the *LIT1* CpG island is conserved between human and mouse (25). This differential methylation is acquired in the germ line (33) making it an excellent candidate for an ICR. Frequent loss of maternal methylation at the *LIT1* CpG island has been observed in BWS patients (40–50%) (34). A targeted deletion of *LIT1* leads to loss of silencing of $p57^{Kip2}$ in both mice and in human cell lines (33,35). Finally, the region between the *Kcnq1* and $p57^{Kip2}$ shows both physical and mechanistic similarities to the imprinted domain containing *Igf2r* and its antisense control transcript, *Air* (36).

We, therefore, investigated whether the similarity in phenotypes between BWS and TNDM is a reflection of a regulatory association between *ZAC* and $p57^{KIP2}$.

MATERIALS AND METHODS

Cell lines

Human ovarian cancer (HOC) cell lines (PA-1 and SKOV-3) were used in this study. The source of these cells is as described previously (37). They were grown in either DMEM or RPMI1640 supplemented with 10% fetal bovine serum.

Bisulfite PCR methylation assay

Genomic DNAs from primary leucocytes from 17 TNDM patients, 8 BWS patients and 2 normal individuals were prepared as described previously (20). DNA (0.3μg) was digested with EcoRI. Bisulfite treatment was carried using the EZ DNA methylation kit (Zymo research) according to the manufacturer's instructions. PCR was carried out using the following primers. Primes sequences for the PCR: For *ZAC*: BS2F (ZAC), 5′-GTTTTTTATGTGTGATTGGGTTTTGGYGG-3′ and BS2R (ZAC), 5′-AAAACRCTAAAACCCCTAACRAAAAC-3′. For *LIT1*: BS2F (LIT1), 5′-TAGGATTTTGTTGAGGAGTTTTTTGG-3′ and BS2R (LIT1), 5′-CCACCTCACACCCAACCAATACCTCACATAC-3′. PCR condition was as follows: denaturation at 95°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 30 s for 30 cycles. PCR products were digested with the appropriate restriction enzyme (for *ZAC*: BssHII and RsaI; for *LIT1*: AciI and MaeIII) and electrophoresed on 2.5% agarose gels. PCR products were subcloned into the TA-TOPO cloning vector (Invitrogen) and sequenced.

Southern blotting

The methylation status of the *LIT1* CpG island was assessed by Southern blotting. Genomic DNAs were digested with BamHI alone or with the methylation sensitive enzyme NotI and electrophoresed on a 0.8% agarose gel, transferred onto Hybond-N^+ filter and fixed by UV cross-linking. The filter was hybridized with the *LIT1* CpG island 1.5 kb probe as described previously (25). Hybridization was carried out at 65°C overnight in Church-Gilbert buffer. Filters were washed with 0.1× SSC, 0.1% SDS at 65°C.

For the transient transfection assay, we examined the methylation status of the transfected plasmids by Southern blotting. Genomic DNAs from the transfected cultured cells (unmethylated and methylated plasmids) were prepared and digested with BamHI and XbaI with or without the methylation sensitive enzyme, HpaII. The plasmids were used as the hybridization probes. Blotting and washing were described as above.

In situ hybridization analysis

Mouse cDNA clones for *Zac1* and $p57^{Kip2}$ were used to prepare sense and antisense RNA by *in vitro* transcription using the DIG RNA labeling kit (Boehringer Mannheim). Sagittal and transverse sections of 8 μm from mouse embryos and placentas at E12.5 were used for *in situ* hybridization, essentially as described previously (38). Sections were counter stained with 4% eosin.

Band shift assay

Oligonucleotides were synthesized for the human $p57^{KIP2}$ CpG island including one putative ZAC binding site (accession number AC005950 and position of sequence 89690–89770) and for the *LIT1* CpG island including three putative ZAC binding sites (accession number U90095 and position of sequence 67890–67810). Where stated, the oligonucleotides were methylated with SssI methylase (New England Biolabs). Nuclear extracts were prepared from 293T cells transfected with the pAc5.1/V5-His, an expression vector (Invitrogen), according to the method of Schreiber *et al*. (39). For a band shift assay, the unmethylated or methylated double-strand DNA oligonucleotides (0.2 ng) were end-labeled with [γ-^{32}P]ATP and were incubated on ice for 30 min with the nuclear extract in 10 μl binding buffer [20 mM HEPES (pH 7.4), 1 mM EDTA, 3 mM $MgCl_2$, 60 mM KCl, 10 mM 2-mercaptoethanol, 4% glycerol, 0.1% Triton X-100 and 1 μg of poly(dI–dC)]. Excess (molar excess of 20-, 100- or 200-fold) unlabeled competitor oligonucleotides of the *LIT1* and $p57^{KIP2}$ CpG islands, either unmethylated or methylated, were added to the reaction mixture. The DNA–protein complexes were then electrophoresed on 5% acrylamide gels.

The super shift assay was performed by adding 0.2–1.0 μg ZAC antibody (Sigma) after 20 min of preincubation of nuclear extract with labeled unmethylated oligonucleotides of the human *LIT1* CpG island.

Methylation-dependent transcriptional repression assay

To test the effect of DNA methylation on the human *LIT1* CpG island we used a transient transfection assay in HeLa cells. A fragment containing the *LIT1* CpG island was generated by PCR using primers (F and R as shown in Figure 3) bearing specific restriction sites at their 5′ and 3′ ends. The primer sequences were as follows: F, 5′-ACTTGAAGGAAAGCAGGCAGGCAGGCAGGAT-3′; R, 5′-CCAACTGGAAGTTTGAGTGGAGTCCTGTTG-3′. PCR products were digested, gel-purified and subcloned into the pGL3-Promoter Firefly Luciferase reporter vector (Promega). Fragments LIT-W (869 bp NotI–HpaI), LIT-X (411 bp NotI–SacI), LIT-Y (458 bp SacI–HpaI) and LIT-Z (387 bp HpaI–R) were generated using the restriction sites present within the *LIT1* CpG island. Plasmids were prepared using a midi prep kit (Qiagen). *In vitro* DNA methylation was performed by incubation with CpG methylase. DNA constructs (2 μg) were transfected into HeLa cells, cultured for 22 h, lyzed and luciferase reading assayed. Firefly luciferase values were normalized against a co-transfected Renilla luciferase reporter, as described in the DLR assay protocol (Promega). Each construct was tested in triplicate in each experiment and the experiment was repeated three times.

Transactivation luciferase assay

The fragment used in the transcriptional activation assay was cloned into PicaGene Enhancer Vector 2 (NIPPON GENE). This fragment corresponds to LIT-X (411 bp) from within the *LIT1* CpG island. It was used unmethylated or *in vitro* methylated (LIT-X me). The human *ZAC* cDNA fragment was cloned into the pSG5 expression vector (Promega). DNA constructs were transfected into cultured cells with the reporter genes using Lipofectamine (Invitrogen). Samples were taken after 48 h, lyzed and luciferase readings taken. Firefly luciferase values were normalized against the control luciferase reporter vector.

RESULTS

Comparison of the expression pattern of the mouse *Zac1* and $p57^{Kip2}$ genes

Zac1 and $p57^{Kip2}$ appear to be expressed in many of the same tissues (7,20,40,41). Given the precedent for co-ordinated regulation of imprinted genes (4), we decided to investigate this more rigorously. We first compared the expression patterns of *Zac1* and $p57^{Kip2}$ in adjacent sagittal sections of E12.5 (embryonic day 12.5) mouse embryos. The genes showed a strikingly similar expression pattern in many tissues. There was strong expression in the lung, tongue, sclerotome, telencephalon and the labyrinth layer of the placenta (Figure 1A, B and D). Both genes were also expressed in Rathke's pouch (Figure 1D), the lumen of cardiac ventricle and bronchus of the lung (Figure 1E) and the epithelium of the adult ovary (Figure 1F). This is the developmental origin of ovarian cancers. We detected some differences in the pattern of expression of the two genes. $p57^{Kip2}$ was detected in the neural epithelium (Figure 1G), the equatorial region of the lens (Figure 1H) and retina (Figure 1I), the nasal epithelium (Figure 1J) and mesonephrum (Figure 1K) where expression of *Zac1* was absent.

The ZAC protein binds within the *LIT1* CpG island

The striking similarity in expression between mouse *Zac1* and $p57^{Kip2}$ raises the possibility of an interaction between two genes. The presence of a zinc finger motif within the ZAC protein suggests a DNA-binding capability. The consensus binding sequence for the ZAC protein has been identified as GGGGCCTC (17). We found that the CpG island of the human $p57^{KIP2}$ gene contains two ZAC binding sites (Figure 2A and B). Given the proposed role for the *LIT1* transcript in regulating the expression of $p57^{KIP2}$, we also looked for ZAC binding sites within the *LIT1* CpG island and found eight potential binding sites (Figure 2A and B).

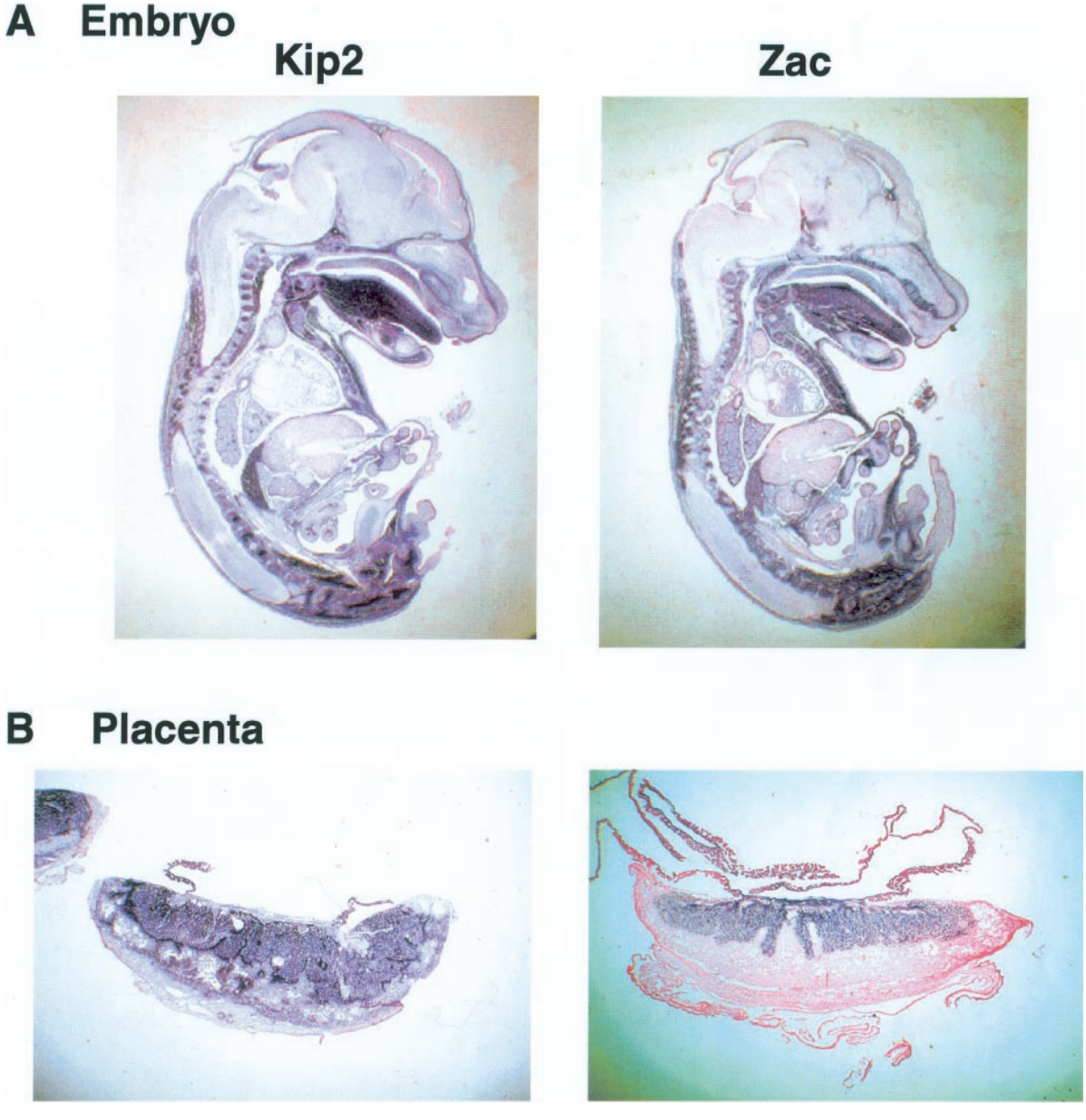
We used an electromobility gel shift assay to ask whether the human ZAC protein could bind either of these regions.

Cell extracts containing the ZAC protein were incubated with the *LIT1* and $p57^{KIP2}$ CpG island oligonucleotides containing the putative ZAC binding sites. The probes were either unmethylated (M−) or methylated (M+). A band shift analysis was performed (Figure 2C). The ZAC protein bound preferentially to unmethylated, a portion of the *LIT1* CpG island probe that contained the putative ZAC binding sites. No detectable binding was observed to the $p57^{KIP2}$ CpG island probe (data not shown). The binding was specific as only the unlabeled, unmethylated *LIT1* CpG island probe competed out the binding and not the $p57^{KIP2}$ CpG island probe or the methylated *LIT1* CpG island probe. Furthermore, specificity of this DNA–protein complex was confirmed in a super shift assay using an anti-ZAC antibody (Figure 2D). This suggests that ZAC protein may bind to the *LIT1* CpG island *in vivo* and perhaps indirectly regulate $p57^{KIP2}$ expression.

Methylation-dependent transcriptional repression assay of the *LIT1* CpG island

There is evidence to suggest that the *LIT1* transcript may regulate imprinting of the 11p15.5 domain. This differentially methylated *LIT1* CpG island is conserved between human and mouse (25). The differential methylation is acquired in the germ line (33) making it an excellent candidate for an ICR.

To determine whether the *LIT1* differentially methylated region has the capacity to function as a regulatory element, we tested it in a transient transfection assay in HeLa cells in which a reporter gene (firefly luciferase) was expressed from an SV40 promoter. Unmethylated or methylated fragments from the human *LIT1* CpG island were assayed for their effect



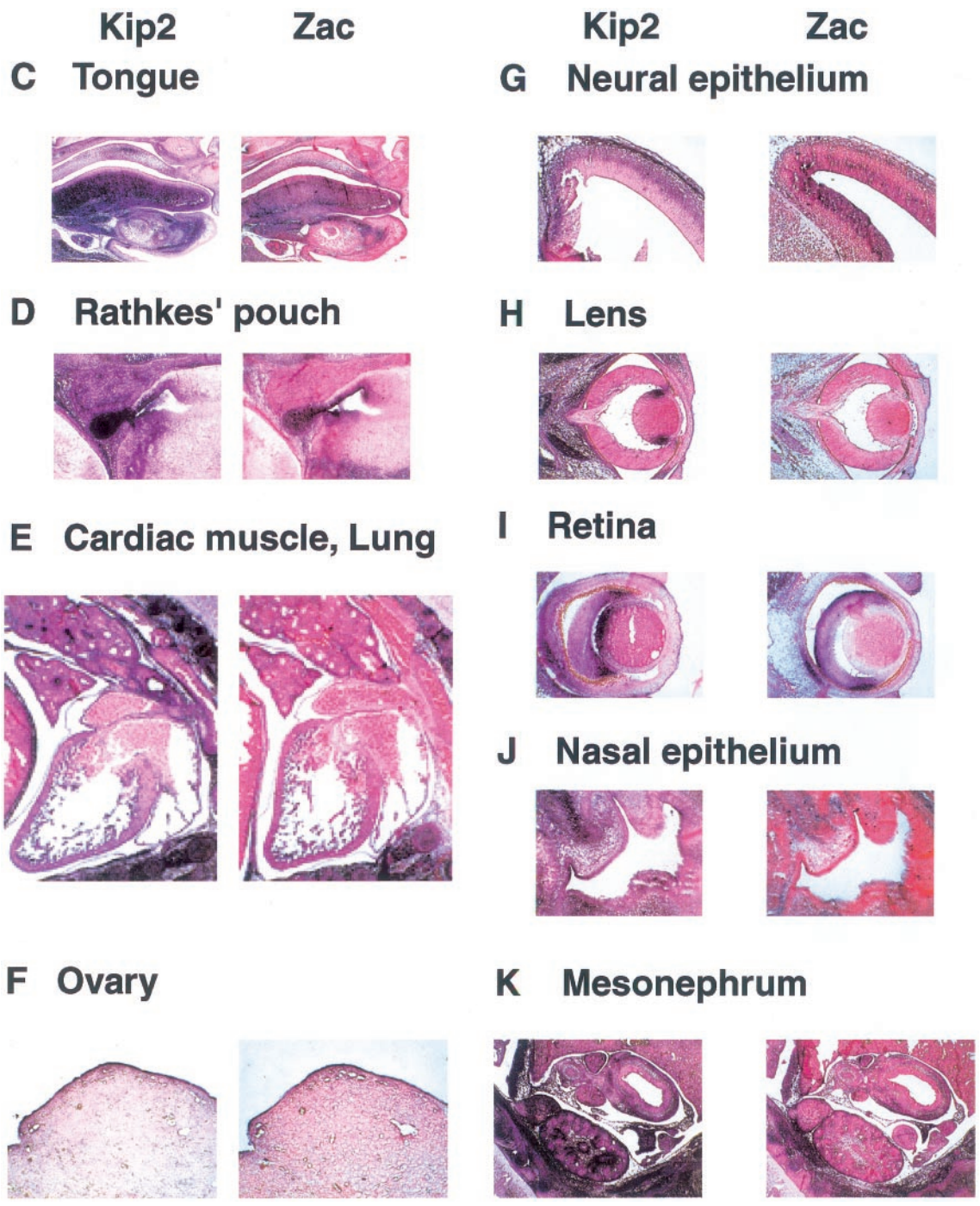


Figure 1. Expression of *Zac1* and $p57^{Kip2}$ in mouse embryos and adult ovary. The comparison of the tissue-specific expression in adjacent sagittal sections of E12.5 embryos (**A**) and placenta (**B**) by *in situ* hybridization. The two genes show striking similarities in their expression patterns. (**C–K**) Detailed analysis of expression during embryonic development of *Zac1* and $p57^{Kip2}$ in transverse and/or sagittal sections of E12.5 embryo. Tongue (C), Rathke's pouch (D), heart and lung (E), telencephalon (G), lens (H), retina (I), nose (J) and mesonephrum (K). Expression of the genes was examined in adult ovary (F).

on transcription of the reporter (Figure 3A). The maintenance of their methylation status after transfection was confirmed by Southern blotting (Figure 3B). Among the fragments we tested, the LIT-X (411 bp NotI–SacI), which contains 57 CpG sites, was shown to act as a strong transcriptional silencer, but only when methylated (Figure 3A). Significantly, the cluster of the ZAC binding sites is present within this region. In contrast, methylation of the LIT-Y fragment, which contains 55 CpG sites, caused negligible repression of the reporter gene (Figure 3A). Interestingly, the LIT-X fragment is contained within a larger fragment (LIT-W) which has a lower repression activity. This may be due to the inclusion of the *LIT1* promoter within the larger LIT-W fragment (M. Oshimura, unpublished data). This promoter may harbor both transcriptional activation and repression properties in this assay. This indicated that LIT-X within the ICR can function

as a *cis*-regulatory element and capable of strong transcriptional silencing in this assay.

Although a large CpG island is present in the 10th intron of *KvLQT1* in both human and mice, its primary sequence is poorly conserved. However, in mice the region containing the DNase I hypersensitive sites within the *Lit1* CpG island, which are upstream of the transcription initiation site, is critical for promoter activity (42,43). The transcriptional initiation site of human *LIT1* gene has been determined in an RNase protection assay and by primer extension analysis by M. Oshimura *et al*. (personal communication). The transcription initiation site is at 67499 nt (U90095) and lies within the *LIT1* CpG island. Also present are two putative CCAAT boxes and a Sp1-binding site but no TATA-box. There is also an AP-1 site downstream of this region. The distal region including F-SacI fragment was previously reported to act as a silencer (44). In mouse, a fragment containing both the hypersensitive sites and the transcriptional start site displayed the strong silencing activity (42). According to the model proposed by Constancia *et al*. (45) repeat sequences may play a role in the methylation of CpG islands. It is interesting that this distal region also contains two direct repeats.

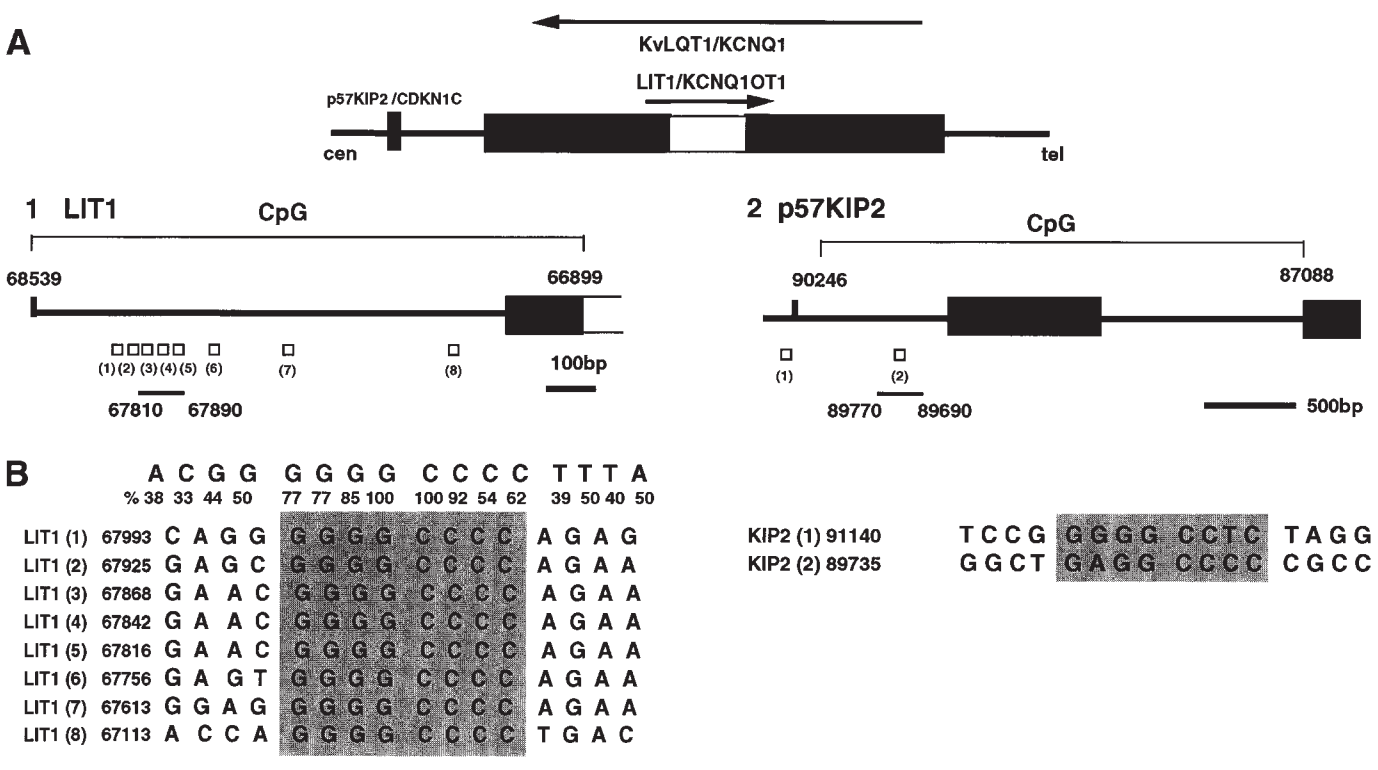
Cell transfection assay

We established an assay to examine whether ZAC binding activates transcription from the *LIT1* promoter. We used two ovarian cancer cell lines (PA1 and SKOV) in which we had shown that endogenous *ZAC* expression was absent (46). These lines were transfected with a luciferase reporter vector with the LIT-X fragment. We also transiently transfected a *ZAC* expression construct into these cells and then measured luciferase activity. *ZAC* behaved as a transcriptional activator (Figure 4) consistent with our binding assay results. Furthermore, when we assayed whether the ZAC protein would activate the methylated *LIT1* reporter in the SKOV ovarian cancer cell line, we did not observe any activation of the reporter suggesting that *ZAC* acts in a signaling pathway only on the unmethylated, paternal *LIT1* allele *in vivo*.

Epigenetic changes at the *ZAC* ICR in BWS and the *LIT1* ICR in TNDM patients

The similarity in phenotypes between BWS and TNDM may reflect a regulatory association between *ZAC* and $p57^{KIP2}$. Our data suggest a possible pathway where over expression of *ZAC*, possibly by loss of methylation of the *ZAC* CpG island, may hyperactivate the *LIT1* gene and silence $p57^{KIP2}$. We therefore examined the imprinting status of *ZAC* in BWS patients and *LIT1* in TNDM patients.

We examined the methylation status of the *ZAC* and *LIT1* DMRs in both types of patients. We performed the restriction enzyme digest and sequencing after PCR of bisulfite-modified genomic DNA (47). After bisulfite treatment of genomic DNA from 8 BWS and 17 TNDM patients, PCR products were digested with AciI and MseIII for the *LIT1* CpG island and RsaI and BssHII for the *ZAC* CpG island (Figure 5A). In this assay, a methylated cytosine will maintain the enzyme recognition site and will be cut, whereas the unmethylated cytocine is converted to thymidine destroying the recognition site. In addition, sodium bisulfite-treated DNA was PCR amplified, subcloned, sequenced and the methylation status was determined at 22 CpG sites in the *ZAC* locus and at 24 CpG sites within the *LIT1* locus. All 8 BWS patients showed normal methylation patterns in *ZAC* (Figure 5B) but 2 TNDM patients



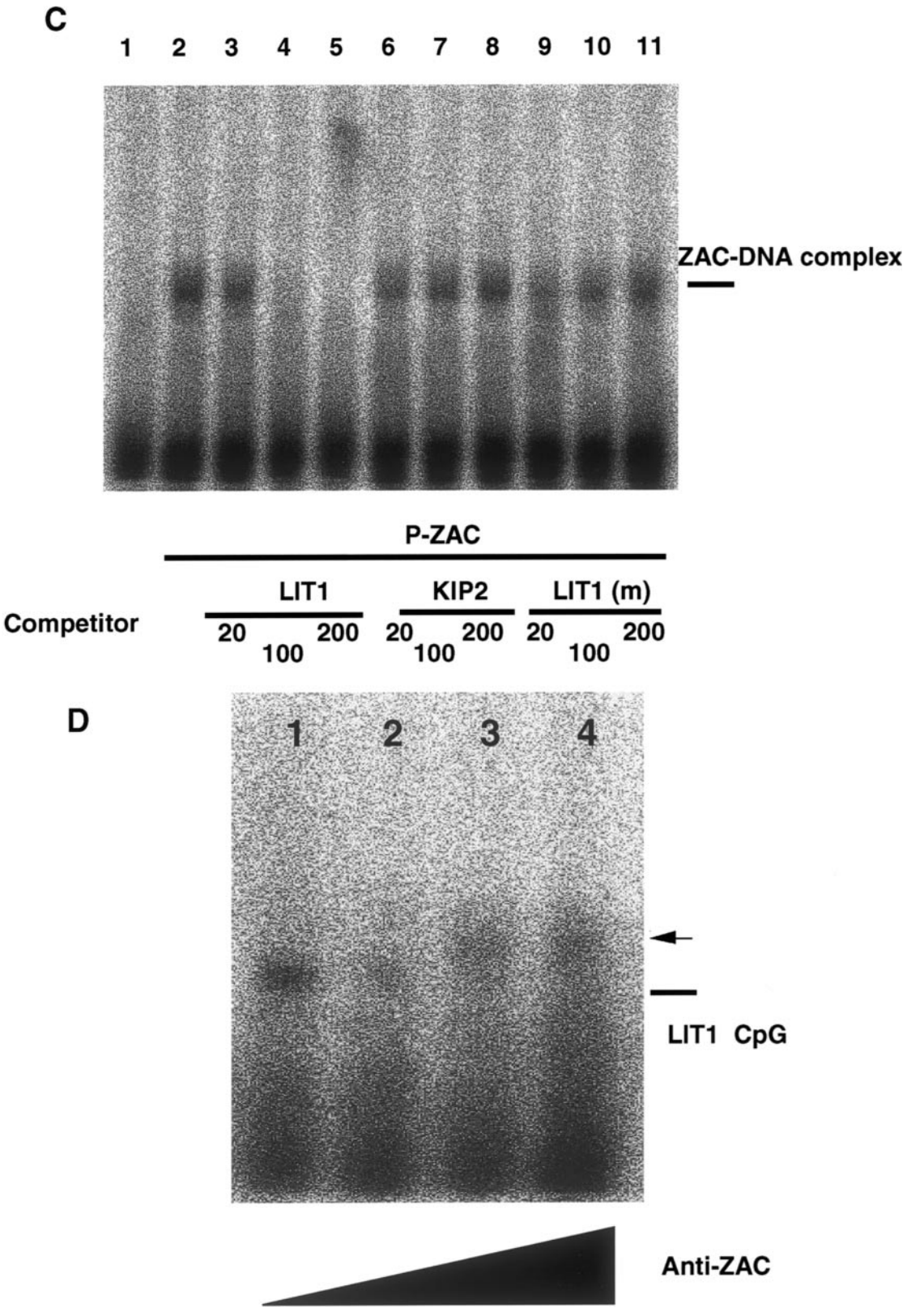


Figure 2. ZAC binds to the *LIT1* CpG island. (**A**) Map of the ZAC binding sites clustered in the *LIT1* CpG island and the two sites in the $p57^{KIP2}$ CpG island. Open boxes indicate putative ZAC binding sites. (**B**) Sequences of the ZAC binding sites within *LIT1* and $p57^{KIP2}$ CpG island. (**C**) Band shift of DNA complexed with ZAC protein. [γ-^{32}P]ATP-labeled oligonucleotides containing CpG islands from *LIT1* were incubated without recombinant ZAC protein (lane 1) and with an ZAC protein (lanes 2–11). The *in vitro* methylation of CpG sequences (me) was performed with SssI methylase. Various cold (unlabeled) competitors of *LIT1*, *KIP2* and *LIT1* (me) oligonucleotides were used in excess: 20-fold molar excess (lanes 3, 6 and 9), 100-fold (lanes 4, 7 and 10) and 200-fold (lanes 5, 8 and 11). (**D**) Specificity of the ZAC protein–*LIT1* CpG island probe complex was confirmed in a super shift assay using an antibody to ZAC; 0 µg (lane 1), 0.2 µg (lane 2), 0.5 µg (lane 3) and 1.0 µg (lane 4).

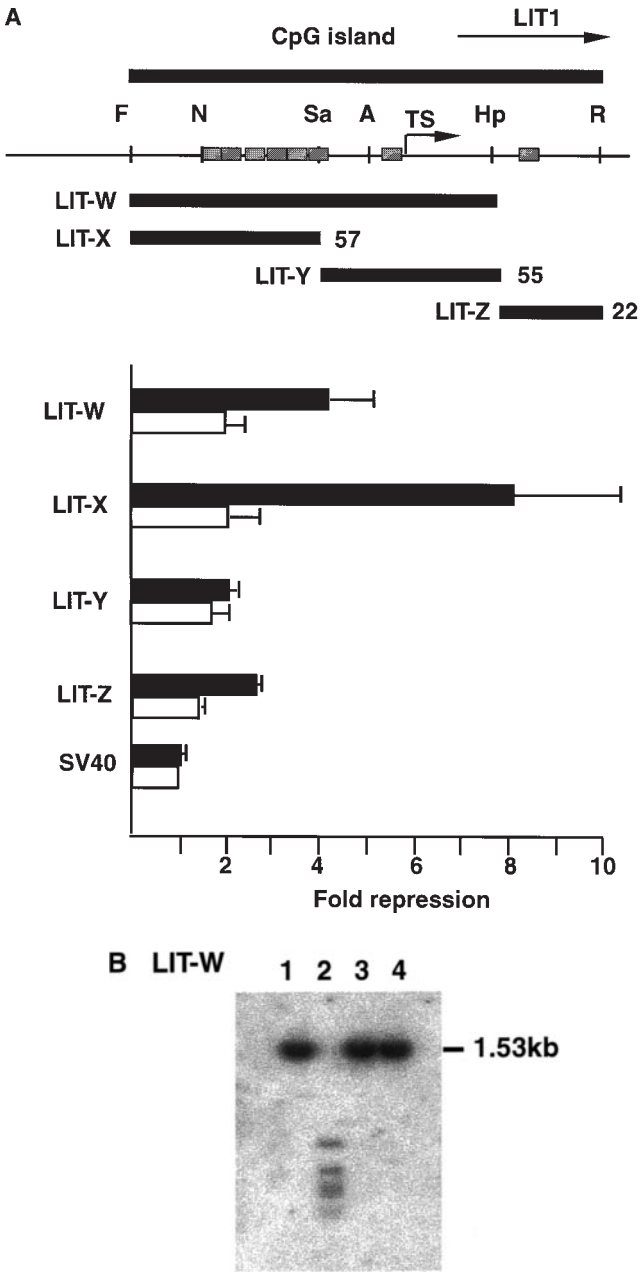


Figure 3. Methylation-dependent transcriptional repression by the *LIT1* CpG island. (**A**) The map indicates the regions of human *LIT1* CpG island tested in the transfection assay. The number of CpG dinucleotides present in each fragment is indicated. The light emission obtained from the SV40 promoter alone, either unmethylated (open bars) or after methylation with SssI methylase (closed bars) was normalized to a value of one and the fold repression of *LIT1* CpG island-containing constructs was calculated relative to these values, shown in the graph. All firefly luciferase values were normalized against a co-transfected Renilla luciferase reporter driven by a thymidine kinase (TK-Renilla) promoter. Cells transfected with TK-Renilla alone demonstrated no firefly luciferase activity. Error bars show calculated SEM values for repeated experiments. Gray boxes show the putative binding sites for ZAC. F and R show the locations of the primers to amplify the CpG island, Sa is SacI site; A is AscI site; N is NotI site; Hp is HpaI site. TS is transcriptional start site (M. Oshimura, unpublished data). (**B**) Maintenance of the methylation status after transfection. Genomic DNAs from cells transfected with either unmethylated or methylated plasmid DNA were examined by Southern blotting. DNAs from unmethylated LIT-W transfected cells (lanes 1 and 2) and SssI-methylated LIT-W (lanes 3 and 4) were digested with BamHI and XbaI without (lanes 1 and 3) and with the methylation sensitive restriction enzyme HpaII (lanes 2 and 4).

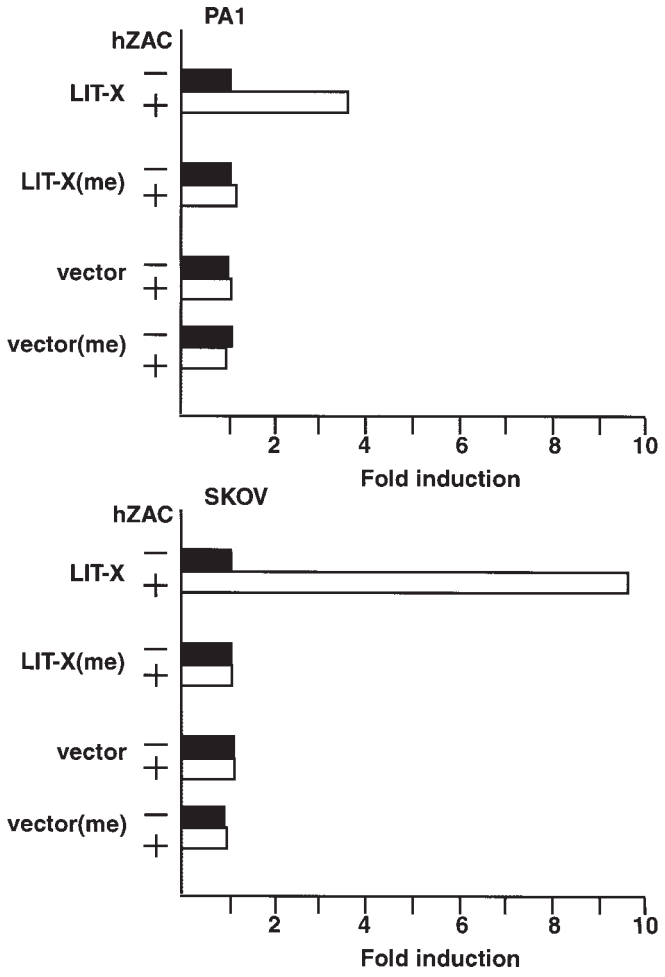


Figure 4. Transcriptional activation of the *LIT1* CpG island by ZAC. Two HOC cells (PA1 and SKOV) were transiently co-transfected either with the unmethylated (LIT-X) or with the methylated [LIT-X(me)] reporter genes without the SV40 promoter in combination with the *ZAC*-pSG5 expression vector. pSG5 vector DNA without the *ZAC* cDNA insertion was used as a control.

showed loss of methylation in the *LIT1* CpG locus (Figure 5B, data shown for AciI). One of these patients exhibited UPD of chromosome 6 (UPD6). The other patient had a normal karyotype and loss of methylation on the maternal *ZAC* DMR region. Sequencing revealed that all 24 *LIT1* CpG sites were predominantly unmethylated in both patients (Figure 5C). The second patient was characterized with TNDM complicated with umbilical hernia and macroglossia, features commonly seen in BWS patients. Finally, we confirmed loss of methylation at the *LIT1* locus in these two patients by a second technique, Southern blotting. (Figure 5D). Both patients showed loss of differential methylation of the *LIT1* locus in association with presumed biallelic expression of *ZAC*.

DISCUSSION

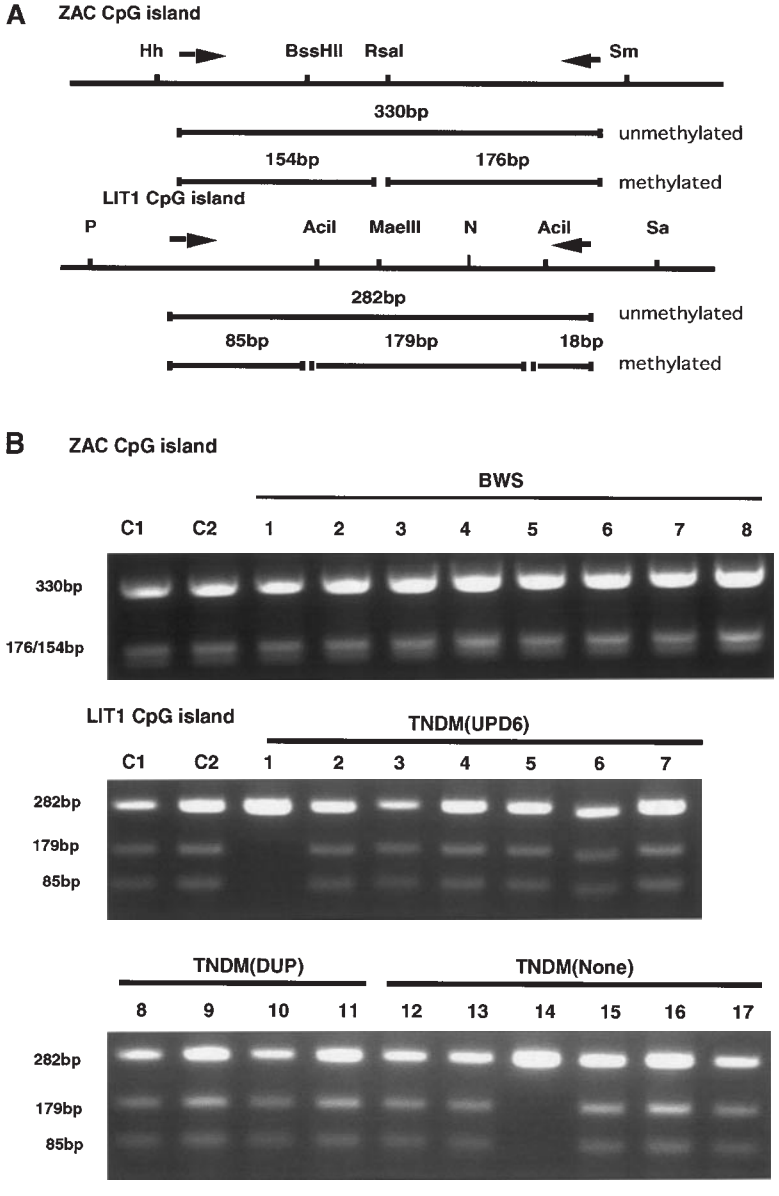
While investigating the function of *ZAC*, an imprinted tumor suppressor gene, we found that this gene has a strikingly similar expression pattern to a second imprinted gene, $p57^{Kip2}$. In humans, *ZAC* is a candidate in TNDM while

p57^{KIP2} has been shown to play a role in BWS. In addition to the similarity in expression, we noticed that some of the features seen in BWS, such as tumors, intrauterine growth abnormalities, umbilical hernias, macroglossia and defects in insulin are also found in TNDM patients metabolism.

We were unable to demonstrate a direct interaction between the ZAC transcription factor and the *p57*^{KIP2} gene. However, we found that ZAC bound to, and activated expression of a third imprinted gene, *LIT1*. *LIT1* has been shown to act an ICR that negatively regulates the *p57*^{KIP2} gene in *cis*. Though few BWS patients have genetic mutations in the *p57*^{KIP2} gene itself, nearly 60% showed loss of methylation of the *LIT1* CpG island. By showing that *ZAC* directly binds to the *LIT1* CpG island *in vitro* and activates transcription, we have identified a possible role for ZAC in the regulation of *p57*^{KIP2}.

We have shown that some patients with TNDM (2 out of 17) have defects in methylation of *LIT1* CpG island, supporting a link between these genes. Furthermore, ZAC has similarities with p53, a known tumor suppressor gene. p53 regulates p21, a member of the same family of cyclin-dependent kinase inhibitors as p57^{KIP2}. Since our data suggest that *ZAC* may regulate *p57*^{KIP2}, the two genes may form part of a signaling pathway for regulating cell cycle progression. p73, a homologue of p53, was suggested as an imprinted gene (48) and more recently has been shown to directly regulate p57^{KIP2} (49). Further work is required to link these genes but our initial conclusion is that we have potentially identified a novel imprinted pathway similar to the *Igf2/Igf2r* pathway.

Finally, our finding that *ZAC* may indirectly regulate *p57*^{KIP2} indicates a potential role for *ZAC* in BWS. While we did not detect changes in DNA methylation at the *ZAC* CpG island in BWS patients, we were only able to survey a small number of patients. We did find changes in *LIT1* DNA methylation in two TNDM patients suggesting a more global defect in imprinted DNA methylation in these patients.



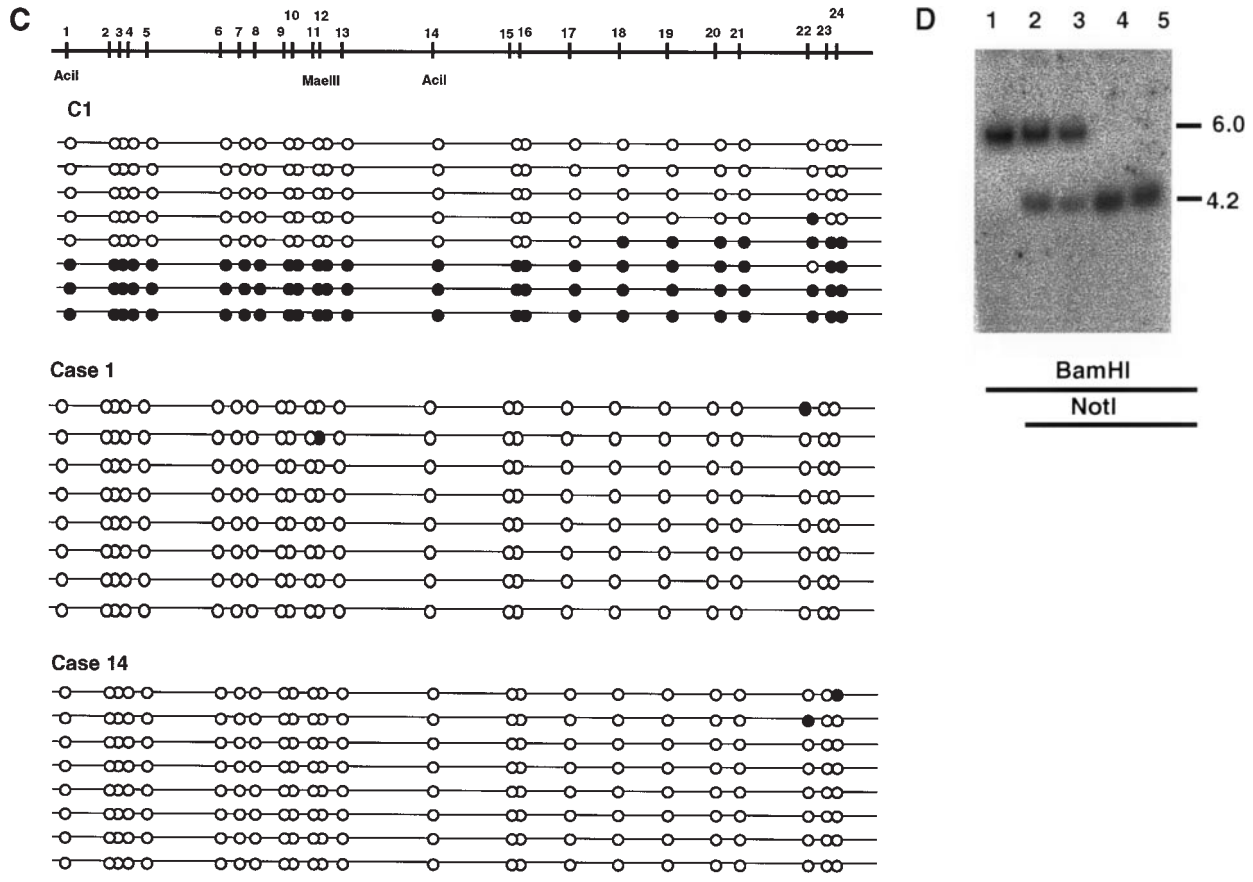


Figure 5. Methylation status of the *ZAC* and *LIT1* CpG islands in BWS and TNDM patients. (**A**) Map of the CpG islands showing the position of the primers used, the methylation sensitive restriction enzyme sites and the predicted product sizes to distinguish between unmethylated (undigested) DNA and methylated (digested) DNA (shown for *ZAC*: AciI; and for *LIT1*: RsaI). (**B**) BWS genomic DNA (top row) is from patients where the methylation status of the *LIT1* CpG island is known: lanes 1–6, unmethylated *LIT1* and lanes 7 and 8, methylated *LIT1*. TNMD genomic DNA (second and third rows) is from previously characterized patients: paternal UPD6 in row 2, lanes 1–7, paternal duplication in row 3, lanes 8–11; and non-UPD/non-duplication in row 3, lanes 12–17. C1 and C2 represent normal controls. All patients with BWS show normal methylation patterns in the *ZAC* CpG island. Two patients with TNDM exhibit an abnormal methylation pattern. (**C**) Bisulfite sequencing analysis (methylation shown as closed circles) showed that 24 CpG sites at the *LIT1* ICR are almost completely unmethylated in 2 TNDM patients (case 1 is paternal UPD6, case 14 has a normal karyotype with an unmethylated ZAC DMR). Case 14 is complicated with umbilical hernia and macroglossia. (**D**) Southern blot analysis of the *LIT1* CpG island in primary leucocyte DNA from the two TNDM patients that showed loss of methylation in the bisulfite assay. The methylation status was analyzed after double digestion with BamHI plus NotI. When the 6.0 kb BamHI fragment encompassing in the *LIT1* CpG island is digested with NotI, a 4.2 kb fragment is produced. Control DNA digested only with BamHI generated a 6.0 kb band (lane 1). Two independent leucocyte DNA samples from normal individuals were found to be a hemi-methylated (lanes 2 and 3). Only the 4.2 kb band is seen in the DNAs from case 1 and case 14 with TNDM confirming loss of DNA methylation (lanes 4 and 5).

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