Characterization of the Methylation-sensitive Promoter of the Imprinted ZAC Gene Supports Its Role in Transient Neonatal Diabetes Mellitus*

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ZAC is a recently isolated zinc finger protein that induces apoptosis and cell cycle arrest. The corresponding gene is imprinted maternally through an unknown mechanism and maps to 6q24–q25, within the minimal interval harboring the gene responsible for transient neonatal diabetes mellitus (TNDM) and a tumor suppressor gene involved in breast cancer. Because of its functional properties, imprinting status, and expression pattern in mammary cell lines and tumors, ZAC is the best candidate so far for both disease conditions. In the present work, we delineated ZAC genomic organization and mapped its transcriptional start site. It is noteworthy that the ZAC promoter localized to the CpG island harboring the methylation imprint associated with TNDM and methylation of this promoter silenced its activity. These data indicate that the methylation mark may have a direct effect on the silencing of the ZAC imprinted allele. Our findings further strengthen the hypothesis that ZAC is the gene responsible for TNDM and suggest a novel mechanism for ZAC inactivation in breast tumors.

The study of imprinted loci is of particular interest in the context of the coordinated regulation of neighboring genes on the megabase scale (1). Moreover, imprinted genes are frequently involved in developmental processes, and loss of imprinting results in a disease condition in many cases (2). The number of imprinted genes is limited (nearly 40 have been identified in human or mouse (37)), and the mechanisms underlying imprinting are as yet poorly understood. Recently, a novel imprinted locus was mapped to human chromosome 6q24–q25 by different strategies. Kamiya and co-workers (3) used restriction landmark genomic scanning to isolate genomic sequences differentially methylated on genomes of maternal and paternal origin, while Gardner and co-workers (4) determined the minimal interval harboring the imprinted gene responsible for transient neonatal diabetes mellitus (TNDM).¹

TNDM is a rare genetic form of diabetes. It is a developmental disease of insulin production (5) predicted to arise from a defect in pancreatic β-cell maturation (6, 7). Most TNDM cases are sporadic, but familial cases also exist. The heritable forms of the disease are paternally inherited (8–10). Several cases of TNDM were reported to be due to paternal uniparental disomy of chromosome 6 (11–13) or partial trisomy of chromosome 6q with duplication of the paternal genome (14–17). Duplications (15) and interstitial deletions (18) of the same region of the maternally inherited chromosome 6 are not associated with the disease. These findings strongly suggest that TNDM results from the overexpression of a paternally expressed, maternally imprinted gene on chromosome 6q. Using polymorphic markers and linkage analysis, the gene responsible for TNDM was localized between markers D6S308 and D6S310 (19, 20). Further studies identified a minimal region containing the gene responsible for TNDM (4). The TNDM locus is 550 kb in length and harbors several CpG islands, of which one (CpG2) is methylated on the maternal allele and unmethylated on the paternal allele (4).² The importance of this CpG island in the etiology of TNDM is further underlined by the discovery that a limited number of patients do not display cytogenetic alterations but rather a defect in the methylation pattern of CpG2 (4). ZAC, encoding a novel zinc finger protein recently isolated in this laboratory (see below) is localized in this region, and Kamiya and co-workers (3) have demonstrated that ZAC is maternally imprinted in several fetal and adult tissues. Its functional properties (induction of cell cycle arrest and apoptosis) are compatible with a role in development and differentiation, and we hypothesized that overexpression of ZAC may be responsible for TNDM.

We recently reported the cloning and functional characterization of ZAC, which encodes a novel zinc finger protein inducing apoptosis and cell cycle arrest (21–23). ZAC was isolated independently by Abdollahi and co-workers (24, 25) in a search for genes in which expression is lost in an in vitro model of cell transformation, hence the name LOT1 for lost on transformation. Interestingly, ZAC/LOT1 was found to map to 6q24–q25 (22, 25), a chromosomal region known to harbor a tumor suppressor gene for many types of solid tumors, including breast and ovary tumors and melanoma (26). Because of its functional properties, chromosomal localization, and loss of expression in a model of cell transformation, we hypothesized

¹ The abbreviations used are: TNDM, transient neonatal diabetes mellitus; bp, base pair(s); nt, nucleotide(s); kb, kilobase(s); ZAC, zinc finger protein that induces apoptosis and cell cycle arrest; RT, reverse transcriptase; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; DMR, differentially methylated region; IC, imprinting center; RPA, RNase protection assay; PAC, P1 artificial chromosome; EST, expressed sequence tag; Me-C, methyl-cytosine.

² D. J. G. Mackay, unpublished observation.
that ZAC may be the tumor suppressor gene on 6q24. ZAC is not inactivated according to the two-hit hypothesis proposed by Knudson (38) for the retinoblastoma susceptibility gene. In contrast, we showed that ZAC is expressed in normal mammary epithelial cells and that 60% of mammary tumor-derived cell lines display a complete loss of ZAC expression (27). The remaining 40% of mammary epithelial cell lines as well as some unselected primary breast tumor samples displayed a down-regulation of ZAC expression. Interestingly, ZAC expression could be reinduced by treatment of some cell lines with azacitidine, an inhibitor of DNA methyltransferase, indicating that methylation of the ZAC promoter may critically regulate its expression. In this context, we undertook the isolation and characterization of ZAC gene to elucidate its imprinting mechanism and pave the way for future studies aimed at testing the actual involvement of this gene in TNDM and breast cancer.

EXPERIMENTAL PROCEDURES

RACE—Rapid amplification of the ZAC cDNA 5′ end was performed from placenta poly(A)+ RNA using the Marathon cDNA amplification kit (CLONTECH, Palo Alto, CA) according to the manufacturer's instructions, except that RT was performed with ZAC-specific primers: 5′-GTGGGCTGTGGTGTTG-3′ and 5′-GTCCTTCTTCCACCACCTCATTCA-3′. RT products were amplified with AP1 primer and different ZAC-specific primers: R1 (5′-GCCATAAGCACACAGACGACAGATG-3′), R3 (5′-TGACGACCAACTCCTGCTTGGCAG-3′), 5′-TGGTCCCAATTGTTTTCCTG-3′), R3 (5′-TGGTCCCAATTGTTTTCCTG-3′), R3 (5′-TGGTCCCAATTGTTTTCCTG-3′), R3 (5′-TGGGCTGTGGTGTTG-3′), and R7 (5′-CGTCCGCTCCGCTGCTG-3′) using the Advantage cDNA PCR kit (CLONTECH). For AP1-R1 and AP1-R2 primer pairs, a nested PCR was subsequently performed with AP2 and R2 (5′-TATGCTGCGCCATGCGG-3′), or R4 (5′-GACGACGACACAGCTTGG-3′), respectively (see Fig. 1 for primer locations). PCR products were digested with NotI, subcloned into pBS (NotI-EcoRV), and sequenced.

RPA and RT-PCR—Total RNAs were prepared from mammaryplasty reduction tissues, cell lines, and placental tissues (23). Pituitary gland poly(A)+ RNA, human fetal liver, and human kidney total RNAs were from CLONTECH, and human total ovary RNA were from Research Genetics, Inc. (Huntsville, AL).

RPA was performed as previously described (27). The RPA antisense ZAC probe was made by T7 in vitro transcription of pBS-3′-5′-876 (see below) digested with NcoI. A positive in vitro T3 transcript sense control was deleted from the same plasmid digested with NotI. 50 μg of total RNA or 0.5 μg of poly(A)+ RNA were hybridized overnight at 56 °C with 2 fmol of gel-purified antisense ZAC probe and treated with RNase A/T1. Protected fragments were analyzed on a 5% acrylamide, 8 M urea gel.

RT was performed from 0.5 μg of poly(A)+ RNA or 1 μg of total RNA with random primers as described previously. 30 cycles of PCR were performed on 0.2 (pituatory) or 2 μl (other tissues) of RT product using P1 (5′-GACGACGACACAGCTTGG-3′) and P2 (5′-CCAAAGGCCATTGGTGCTGCG-3′) oligos (see Fig. 1 for primer locations). Bands were agarose gel-purified, subcloned into pGEM-T (Promega), and sequenced.

RESULTS

The mechanism responsible for ZAC imprinting has not yet been determined. The minimal region for TNDM is covered by clones 468K18, 340H11, 197L1, 3B11, 83M4, and 91J24. Sequence examination revealed the presence of several CpG islands of which one (CpG2) is differentially methylated on the paternal and maternal alleles (4). Until now, the isolated most 5′ exon of ZAC was located ~70 kb centromeric from CpG2. In the present report, we used the 5′ RACE technique to isolate the ZAC most 5′ exons. Using primers annealing to exon IX or VII, we could extend the ZAC sequence through different exons to a CpG-rich region (Exon I). We determined the exon/intron organization of the ZAC gene by aligning the sequences of the RACE clones with NT029260 assembled from PACs 197L1, 340H11, and 468K18. The ZAC gene comprises 9 exons, with exon VIII harboring the translation start site (Fig. 1). This gene organization was confirmed by RT-PCR of total RNA isolated from different tissues including mammary gland and pancreas using primers in exon I and IX (Fig. 2). In addition, alternative splicing of exons II, IV, V, and VIII and the use of alternative splice sites for exons IV and VII were detected. Despite the significant abundance of the various alternatively spliced isoforms of the ZAC 5′ untranslated region, their relevance remains elusive at this point. By contrast, we recently showed that splicing of exon VIII (first coding exon) modulates the functional properties of the corresponding translation products (25). Of the 50 RACE clones sequenced, 35 were incomplete, but 15 had their 5′ ends into CpG2. There were two groups of those RACE clones; 7 were incomplete and had very short 5′ ends extending only ~10–50 nt into exon I, 7 extended between nt 52937 and 52907 of 340H11, and 1 RACE clone (the longest) extended to nt 53070 of 340H11 (note that ZAC is antisense to 340H11). It was impossible to further extend this 5′ end by using primers annealing to exon IV, III, or I. During the course of this study, Hamilton’s group (25) corrected the sequence of the human LOT1/ZAC sequence (U72621), which now extends into the same CpG island to nt 52879 of 340H11. To determine the 5′ boundary of exon I, we performed RNase protection assays using

![Figure 1](https://www.jbc.org/content/18654/4/16854/F1.large.jpg)

**Fig. 1. Genomic organization of ZAC.** Sequencing of RACE clones indicated the presence of 9 exons, for which the sequence coordinates are given according to the numbering of PACs 340H11 and 468K18 (ZAC is antisense to these published sequences): I, 340H11 nt 53070–52754; II, 340H11 nt 29835–29579; III, 340H11 nt 3642–13571; IV, 40H11 nt 0948–10822, 10894–10822, 10876–10822, or 10862–10822, corresponding to alternative splice acceptor sites; V, 340H11 nt 9492–9451; VII, 340H11 nt 5244–5133, 5220–5133, 5206–5133, or 5206–5137 depending on the use of alternative acceptor and donor splice sites; VII, 468K18, 340H11 nt 130411–129936; and IX, 468K18 nt 124614–122253. As there were several transcription start sites (see text and Fig. 3), the size of exon I varied between 160 and 320 bp. Acceptor and donor sites were in good agreement with consensus splice sites (not shown). Exons II, IV, V, and VIII are alternatively spliced (indicated by an asterisk). The translation start site is located in exon VIII. The CpG island is indicated as well as the location of the primers used for RACE (R1–R7) and RT-PCR (P1 and P2). The scale bar concerns the exons only. The sizes of the larger introns are indicated.
mediate fragments (800 bp) corresponded to PCR artifacts. (400–600 bp) corresponded to the larger ones without exon VIII. Intermediate fragments were gel-purified and subcloned, and 40 clones were sequenced. The larger fragments (900–1100 bp) corresponded to different fragments were run together with RT samples were used as negative controls. Molecular weight markers (MW) (not shown) and MDA-MB453 (ZAC cell line) (Fig. 2). We next investigated whether the region upstream of the potential transcription start sites had a promoter activity. We subcloned a 876-bp genomic fragment corresponding to nt 52829–53306 of 340H11, covering part of exon I on its 5' end. Pituitary and mammary gland RNAs gave 2 major protected fragments of 95–105 nt, corresponding to a 5' end between nt 52934–52925 of 340H11 close to the start sites given by the RACE clones extending to nt 52937–52907 of 340H11 (Fig. 3). Two weak, longer protected fragments could be detected around 240–250 nt corresponding to the start site given by the longer RACE clone (nt 53070 of 340H11). No protected fragment was detected in yeast RNA or in RNA from the ZAC non-expressing cell line MDA-MB231. All of these data predicted a length of ZAC cDNAs between 2.9 and 3.6 kb in agreement with the size of the mRNAs we described previously (27).

We next investigated whether the region upstream of the potential transcription start sites had a promoter activity. We subcloned a 876-bp genomic fragment corresponding to nt 53704–52829 of 340H11, in sense (5'–3'–5'-nt 3'–3') orientations relative to ZAC exons, into the pGL3basic reporter gene. Luciferase activity was measured in CAL-51 (ZAC+ cell line) (not shown) and MDA-MB453 (ZAC– cell line) transfected cell lines. As shown in Fig. 4A, this fragment (5'–3'–5'-nt 3'–3') contained a promoter activity directed toward ZAC exons, whereas the fragment in the reverse orientation (3'–5'–5'-nt 3'–3') was devoid of such activity. The results in CAL-51 cells were similar, except that pGL3-C had a 4–5-fold higher activity than in MDA-MB453 (105 ± 7-fold induction over pGL3basic) and 5'–3'–3' a slightly weaker activity (10 ± 1, compared with the MDA-MB453 value, 30 ± 2) (not shown). Using deletion mutants, we confirmed the RPA experiments by showing that the minimal promoter was located between nt 53020 and 52829 of 340H11 in the minimal 192-bp construct, which includes the potential start sites at nt 52934–52925 of 340H11. This promoter is TATA-less and GC-rich, which is in agreement with the multiple transcription start sites we detected.

As we found a promoter activity in CpG2 directed toward ZAC, we next investigated whether this promoter activity was modulated by methylation. We used SssI methylase (CpG methylase), which induced the dense methylation of 5'–3'–3' (73 Me-C in a CpG context), and AluI methylase, which methylates 5 cytosine residues in a CpG context. SssI methylase completely abolished the promoter activity of 5'–3'–3' in both MDA-MB453 (Fig. 4B) and CAL-51 cells (not shown) in contrast to AluI methylase, which had no effect (Fig. 4B). Altogether, these data demonstrated that CpG2 contains the promoter controlling ZAC expression and that its activity is negatively modulated by dense methylation of cytosine in the CpG context.

**DISCUSSION**

Among the different epigenetic modifications involved in imprinting, DNA methylation is particularly relevant, as the great majority of imprinted genes examined so far contain differentially methylated regions (DMRs) on the maternal and paternal alleles (28–30). These DMRs have variable locations within different imprinted genes, and their functional role in the silencing of the imprinted allele is not always indisputable. Some DMRs harbor a promoter activity that is regulated by methylation; our data support such a function for the DMR of the TNMD locus. We have shown here that the ZAC promoter resides in this DMR and that in vitro methylation completely abolishes its activity. These data support a model in which the methylation mark on the imprinted maternal allele represses ZAC transcription, whereas the unmethylated CpG2/ZAC promoter drives the expression of the paternal allele. This is the simplest model of methylation-induced monoallelic expression, where the methylation-induced modification of heterochromatin represses gene transcription. A similar mechanism may be postulated for the imprinting of mZac gene, which is also paternally expressed, as a recent study reported that DNA demethylation but not histone deacetylase inhibition reactivates mZac in uniparental (maternal) mouse embryonic fibroblasts (31). Furthermore, the 5' end of mZac resides in a CpG island with maternal allele-specific methylation analogous to the human gene.3

Other DMRs are involved in establishing differential methylation marks at precise locations within imprinted loci and have been named imprinting centers (ICs) (32). The location of the IC controlling the methylation status of the ZAC promoter remains an open question. Several imprinting models have been described. The expression/competition model is exemplified by the Igf2r locus, where the imprinting center is a DMR located within intron 2. This DMR serves as the promoter for the noncoding antisense Air mRNA and governs the expression

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3 G. Kelsey, personal communication.
TNDM patients have neither paternal UPD6 (uniparental disomy of chromosome 6) nor paternal duplication of 6q24 but instead display a methylation defect in CpG2 (35). We can predict that ZAC is biallelically expressed in these patients. Transgenic mouse models will help to confirm the involvement of ZAC in TNDM. Besides ZAC, the TNDM locus encodes many ESTs, which may correspond to other TNDM candidate genes. One such candidate is HYMAI, a maternally imprinted, apparently non-coding cDNA, which partially overlaps with CpG2, the ZAC promoter and its first exon (36). The reported sequence of HYMAI is sense with regard to ZAC, but according to the annotations of the ESTs used for its cloning it could be antisense. This issue will have to be addressed to find out whether HYMAI encodes a noncoding antisense RNA, as is observed in several imprinted loci, or whether it is co-linear with ZAC and both genes compete for the same promoter.

Because of its antiproliferative properties and chromosomal localization, ZAC is a candidate tumor suppressor gene for breast cancer. Provided that ZAC expression is monoulelic in the mammary gland, a single event (the inactivation of the paternally expressed allele) would be sufficient to completely inactivate ZAC, supporting a tumor suppressor function for this gene. The location of the ZAC promoter, which we report here, paves the way for future studies aimed at resolving this important issue.

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