 Human Molecular Genetics, 2002, Vol. 11, No. 18 2091–2102

Defective *de novo* methylation of viral and cellular DNA sequences in ICF syndrome cells

Qian Tao[1], He Huang[1], Theresa M. Geiman[2], Chai Yen Lim[1], Li Fu[1], Guo-Hua Qiu[1] and Keith D. Robertson[2,*]

[1]Tumor Virology Laboratory, Johns Hopkins Singapore, Level 5 Clinical Research Center (MD11), NUS, 10 Medical Drive, Singapore 117597 and [2]Epigenetic Gene Regulation and Cancer Section, NCI, NIH, Building 41, Room C306, 41 Library Drive, Bethesda, MD 20892, USA

Received April 10, 2002; Revised and Accepted June 24, 2002

ICF syndrome (immunodeficiency, centromere instability and facial anomalies) is a recessive human genetic disorder resulting from mutations in the DNA methyltransferase 3B (*DNMT3B*) gene. Patients with this disease exhibit numerous chromosomal abnormalities, including anomalous decondensation, pairing, separation and breakage, primarily involving the pericentromeric regions of chromosomes 1 and 16. Global levels of DNA methylation in ICF cells are only slightly reduced; however, certain repetitive sequences and genes on the inactive X chromosome of female ICF patients are significantly hypomethylated. In the present report, we analyze the molecular defect of *de novo* methylation in ICF cells in greater detail by making use of a model Epstein–Barr virus (EBV)-based system and three members of the unique cellular cancer–testis (C–T) gene family. Results with the EBV-based system indicate that *de novo* methylation of newly introduced viral sequences is defective in ICF syndrome. Limited *de novo* methylation capacity is retained in ICF cells, indicating that the mutations in *DNMT3B* are not complete loss-of-function mutations or that other DNMTs cooperate with DNMT3B. Analysis of three C–T genes (two on the X chromosome and one autosomal) revealed that loss of methylation from cellular gene sequences is heterogeneous, with both autosomal and X chromosome-based genes demonstrating sensitivity to mutations in *DNMT3B*. Aberrant hypomethylation at a number of loci examined correlated with altered gene expression levels. Lastly, no consistent changes in the protein levels of the DNA methyltransferases were noted when normal and ICF cell lines were compared.

INTRODUCTION

ICF syndrome (immunodeficiency, centromere instability and facial anomalies) is a rare autosomal recessive disorder caused by mutations in the *DNMT3B de novo* DNA methyltransferase gene (1–3). The *DNMT3B* gene (4,5) and the ICF susceptibility locus (6) have been independently mapped to human chromosome 20q11.2. Most ICF patients are compound heterozygotes for their *DNMT3B* mutations, and, with one exception, all of these mutations occur within the C-terminal catalytic domain of DNMT3B and likely fully or partially impair catalytic activity (2,7). Features of this disease include severe immunodeficiency with an absence or profound reduction in at least two immunoglobulin isotypes and in some cases impaired cellular immunity, neurologic and intestinal dysfunction, peculiar facial features, and delayed developmental milestones. Affected individuals usually suffer from severe respiratory tract infections, and often do not survive into adulthood (8,9).

Recent studies showed that ICF cells are extremely sensitive to ionizing radiation despite possessing intact cell cycle checkpoints (10), and that many of the genes whose expression is dysregulated in ICF cells are in some way related to lymphoid cell development or function (11). Cytologically, primary and cultured cells from ICF patients exhibit a marked elongation of juxtacentromeric heterochromatin, primarily affecting chromosomes 1 and 16, and, to a much lesser extent, chromosome 9. Abnormalities include multiradial chromosomes comprised of multiple arms (ranging from 3 to 12) of the above-mentioned chromosomes, whole-arm chromosome deletions or duplications, isochromosomes, centromeric breakage, translocations, and telomeric associations (8,9,12).

At the molecular level, one of the most consistent features of ICF syndrome is the hypomethylation of juxtacentromeric repeat sequences on chromosomes 1, 9 and 16 (13). Interestingly, these chromosomes contain the largest blocks of classical satellite long tandem repeat arrays (satellite 2 for chromosomes 1 and 16, and satellite 3 for chromosome 9) adjacent to their centromeres (13,14). These regions are normally heavily methylated in somatic cells, and such methylation is likely essential for proper centromere structure

*To whom correspondence should be addressed. Tel: +1 3015948055; Fax: +1 3014964951; Email: robertk@mail.nih.gov

and stability, and chromosome separation during mitosis (12,13). Bisulfite genomic sequencing of satellite 2 repeats revealed a reduction in methylation from ~70% in normal lymphoblasts, to 20% in ICF cells (15). Although there is a drastic loss of methylation from satellite sequences in ICF patients, the overall reduction in cellular 5-methylcytosine levels is relatively small. No detectable loss of methylation in cultured ICF EBV-immortalized lymphoblastoid cell lines (LCLs) was noted, and only a 7% decrease in DNA methylation levels in primary ICF brain tissue was detected, supporting the notion that mutations in *DNMT3B* lead to very selective losses of methylation from the genome (12,16). Other regions that have been shown to become hypomethylated in ICF patients include the non-satellite repeats *D4Z4* and *NBL2* (16), α-satellite repeats, *Alu* repeats, the imprinted gene *H19* (17), and a number of genes (*G6PD*, *SYBL1*, *AR* and *PGK1*) on the inactive X chromosome of female ICF patients (18). Advanced replication time and biallelic expression of several genes on the inactive X chromosome was also noted in the latter study (18). Interestingly, however, a recent microarray analysis that identified a number of genes whose expression is aberrantly up- or downregulated in ICF cells showed that there were no detectable methylation differences in these same genes (11), further underscoring the complexity and our lack of understanding of how *DNMT3B* mutations give rise to the ICF phenotype.

In order to gain a better understanding of the types of DNA sequences whose methylation is established and/or maintained by DNMT3B, we analyzed a number of viral and cellular genes in two extensively characterized ICF cell lines (12) using both bisulfite modification analysis of DNA to examine methylation changes (19) and semiquantitative reverse transcriptase (RT)–PCR to monitor changes in gene expression. We made use of the fact that all ICF lymphoblastoid cell lines (LCLs) were established by *in vitro* infection with Epstein–Barr virus (EBV), a lymphotrophic γ-herpesvirus that infects and immortalizes B cells with high efficiency but does not generally lead to gross chromosomal abnormalities or to cellular transformation (20). Furthermore, the EBV minichromosome (or episome) is an extremely well-characterized system because it has been completely sequenced and its gene expression patterns upon infection and establishment of latency have been extensively studied (20–22). The EBV genome is replicated and its genes transcribed largely by cellular factors in addition to one or more virally encoded proteins. Perhaps the most useful feature of EBV biology is that its methylation status has been extensively characterized and it is known to undergo defined *de novo* methylation events during the establishment of LCLs *in vitro*, thus allowing us to directly study defects in *de novo* methylation in cells lacking a functional *DNMT3B* gene (23–25). Our results show that several EBV gene promoters exhibit significant methylation differences between ICF and normal LCLs, with concomitant changes in levels of viral gene expression, in those regions known to undergo *de novo* methylation as part of the EBV immortalization program (25,26). In fact, all studied EBV sequences expected to undergo *de novo* methylation were hypomethylated in ICF cells, indicating a global defect in *de novo* methylation. A number of cellular DNA sequences have been shown to be hypomethylated in ICF relative to normal cells (16,18,27); however, these sequences were also subject to developmental and lineage-specific influences on DNA methylation pattern establishment, which is a highly complex process. EBV infection and subsequent *de novo* methylation should therefore provide a 'snapshot' of the methylation defect in ICF cells present in the initial primary cell culture without these complicating influences. Further analysis of several cellular cancer–testis (C–T) gene promoters, whose CpG island promoters are known to be hypermethylated in normal cells (28), revealed selective losses of methylation and gene reactivation in ICF cells. Thus, these studies provide the first direct evidence for defective *de novo* methylation in ICF cells, despite the presence of at least one other functional *de novo* methyltransferase [DNMT3A (29,30)], and shed additional light on the types of DNA sequences methylated by DNMT3B. This work also illustrates the utility of the EBV-based system for studying *de novo* methylation.

RESULTS

Global defects in *de novo* DNA methylation in ICF syndrome cells using an EBV-based model system

We have utilized two well-characterized ICF cell lines, termed ICF 1 and ICF 2 (8,12,31). ICF 1 has a homozygous V726G mutation in *DNMT3B* and ICF 2 is heterozygous for an A603T mutation and an intron 22 G-to-A mutation resulting in a three-amino-acid insertion (STP) in DNMT3B (12). As a comparison, the normal parents (referred to as normal male and female) of ICF 2, and in some cases an unrelated normal LCL, were used (31). We utilized a combination of bisulfite genomic sequencing (BGS) (19) and methylation-specific PCR (MSP) (32) to monitor differences in the methylation status of four EBV gene promoter regions and RT–PCR to examine changes in expression of the associated genes. Numerous studies have shown that virtually all genes in EBV virion DNA are hypomethylated (33–35; Q. Tao and K.D. Robertson, unpublished data) yet the episomally maintained viral DNA in established LCLs is hypermethylated in a region-specific manner (24,25), making EBV infection an ideal system in which to study *de novo* DNA methylation events in cells defective for DNMT3B. Two EBV latency promoters (Cp and Wp) were examined (Fig. 1A). These promoters are extremely well characterized in terms of their methylation status, and previous elegant work has shown that Wp, which is the first promoter active upon EBV infection, is initially completely hypomethylated (21,25). Wp drives expression of a viral transactivator termed EBNA-2, which binds to a region upstream of Cp (Fig. 1A), via a cellular protein termed CBF1 (36,37). Cp then becomes active, Wp expression is shut down, and critical regulatory elements within Wp become *de novo* methylated (25). MSP methylation analysis of the critical regulatory regions of Cp and Wp (Fig. 1B) revealed that both were significantly hypomethylated in ICF cells relative to their normal counterparts, although the detection of Cp methylation in the normals was somewhat unexpected since previous reports showed that Cp was unmethylated in LCLs by the less sensitive BGS assay (25). The MSP results for Cp and Wp are quantitated and summarized in Figure 1C. RT–PCR

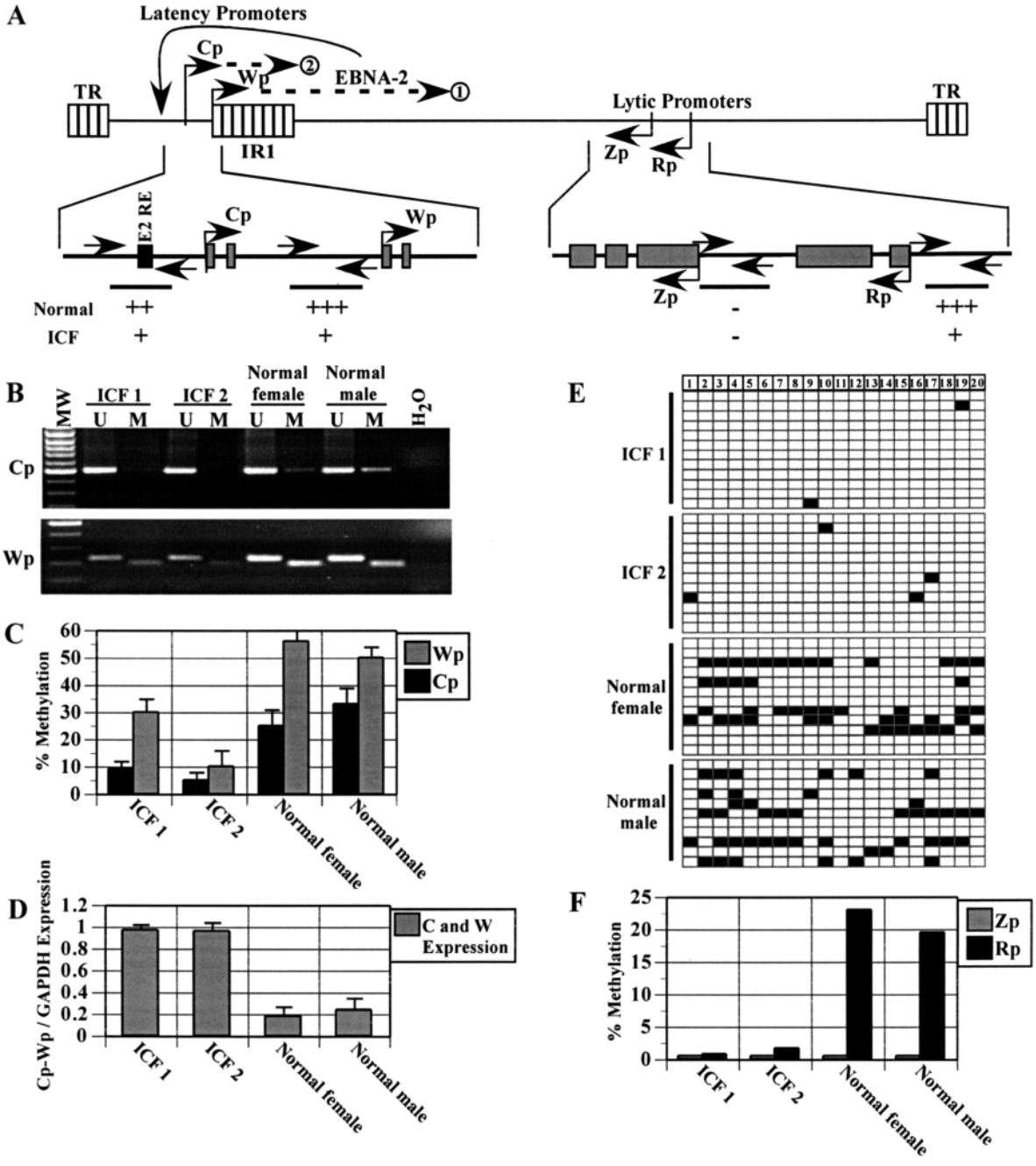

Figure 1. (**A**) Schematic of the EBV genome in linear form, with the relevant promoters under study indicated with bent arrows. Gray boxes are exons and horizontal arrows indicate the regions analyzed for methylation status. 'TR' are the terminal repeats by which the linear virion DNA becomes circularized upon infection. Upon infection of B cells in culture, the hypomethylated virion DNA first initiates transcription from the W promoter ('1', Wp). The mRNA generated from this promoter encodes a viral transactivator (EBNA-2) that binds to a region upstream of Cp (E2 RE) and activates Cp with concomitant shutdown of Wp and establishment of viral latency ('2'). The lytic promoters Zp and Rp are the first to be activated during lytic infection, and are usually active in lymphoblastoid cell lines (LCLs), since spontaneous low-level lytic reactivation is known to occur. A summary of the methylation status of each region (indicated with +/−) in normal and ICF LCLs derived from (C) and (F) is also shown below. (**B**) MSP methylation analysis of ICF and normal LCLs for the Cp (top) and Wp (bottom). 'U' and 'M' indicate amplification of unmethylated or methylated alleles, respectively. (**C**) Quantitation of MSP data from (B) represented as the percentage methylation [M/(U + M) signals]. (**D**) Expression from Cp and Wp, monitored collectively from downstream exons common to both promoters, by semiquantitative RT–PCR. Results of these amplifications are expressed relative to a control amplification for GAPDH mRNA. (**E**) Bisulfite genomic sequencing (BGS) analysis of Rp in ICF and normal LCLs. Twenty CpG sites (numbered across the top of the table) were within the region analyzed. White boxes represent unmethylated CpGs and black boxes represent methylated CpGs. Each row in the grid is an independently cloned and sequenced PCR product. Zp was completely unmethylated at all sites in all four cell lines (not shown). (**F**) Quantitation of BGS data from (E) represented as the percentage methylation at all CpG sites. (**G**) Expression from Zp and Rp by semiquantitative RT–PCR relative to a control amplification for GAPDH mRNA. Results of all PCR reactions are presented as the mean of triplicate experiments ± SD, and were within the linear range of PCR amplification. The result of a single representative experiment is shown in (B). (**H**) RT–PCR expression analysis of an early-lytic-cycle EBV transcript (BHRF1) and a late-lytic-cycle EBV transcript (BLLF1), demonstrating that the ICF LCLs have reduced lytic activation relative to the normal LCLs.

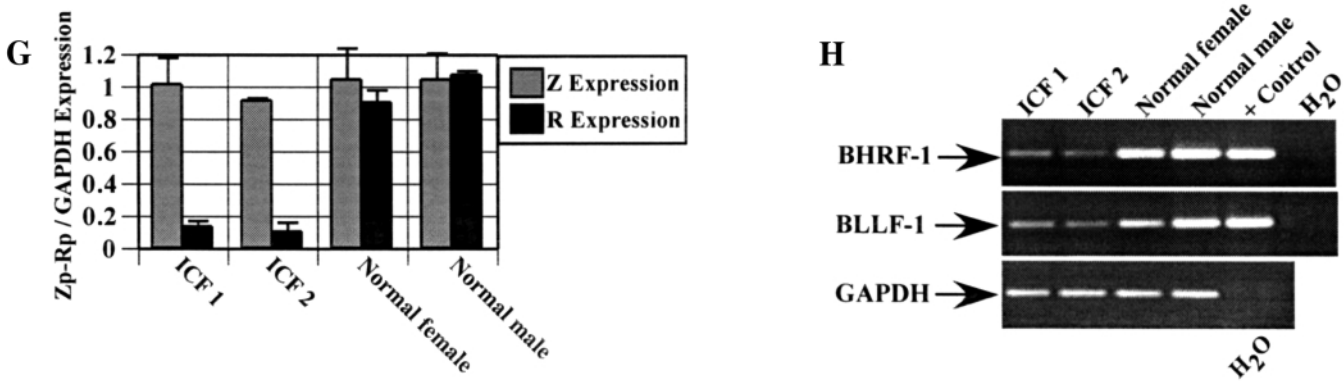

Figure 1 *continued.*

analysis (Fig. 1D) revealed a 4–5-fold increase in expression from these two promoters in the ICF cells. The primers employed in this analysis are downstream of Wp, and are therefore capable of detecting transcripts originating from both Cp and Wp. The increase in transcript levels most likely results from hypomethylation of Wp, since Cp is known to be active in all LCLs and Wp showed the greatest degree of hypomethylation in the ICF cells. Cp and Wp are alternative usage promoters and drive expression of the same set of six viral latency antigens expressed in LCLs (21). Hypomethylation and expression of either promoter will therefore suffice for expression of these viral proteins.

We next analyzed two EBV immediate early lytic gene promoters: Zp and Rp (Fig. 1A). Zp is the first promoter to be activated upon induction of the lytic cycle, and drives expression of the immediate early gene *BZLF1*, a viral transactivator. BZLF1 binds to regions upstream of Rp and in turn activates expression of Rp to produce BRLF1, which goes on to activate other genes involved in the production of infectious virus particles (38–40). In some instances, expression of BRLF1 alone can initiate the lytic cascade (41,42). The EBV genome in LCLs exists predominantly in the latent, episomal state, but, by mechanisms that remain unclear, may undergo low-level spontaneous lytic cycle activation (40). BGS was employed to examine the methylation status of individual CpG sites on cloned DNA molecules from the Rp and Zp regions shown in Figure 1A. Rp was highly hypomethylated in the ICF LCLs relative to the normals (Fig. 1E and F). Some heterogeneity between cloned DNA molecules could be seen, which has been noted previously for other EBV promoters (25,26,43,44); however, clear differences could still be discerned between the normals and the ICF cells. Interestingly, expression of BRLF1 was directly correlated with Rp hypermethylation, in that BRLF1 was more highly expressed (8–9-fold) in the normal controls than in the ICF cells (Fig. 1G). To determine whether the higher expression level of Rp resulted from higher levels of lytic infection in the normal LCLs compared with the ICF LCLs, the expression of two other lytic genes—an early lytic transcript (BHRF1) and a late lytic transcript (BLLF1)—was examined by RT–PCR. Results showed that there was indeed more lytic cycle-associated gene expression in the normal LCLs (Fig. 1H), consistent with the higher Rp expression levels. No methylation was detected within the Zp region in any of the cell lines examined (Fig. 1F and data not shown), indicating that this gene may be controlled by other mechanisms in LCLs. We could detect expression of BZLF1 by RT–PCR at nearly equivalent levels in all cell lines (Fig. 1G). Analysis of the Zp region also demonstrates that the EBV genome is not becoming globally hypermethylated upon infection, but rather is methylated in a region-specific manner—much in the way the mammalian genome is regionally methylated. Overall, the methylation data derived from the four EBV promoter regions is highly consistent (summarized in Fig. 1A). All promoters that are subject to *de novo* methylation (Wp, Cp and Rp) in normal LCLs are highly hypomethylated in ICF cells, indicating that the ICF-specific mutations in *DNMT3B* lead to a global defect in *de novo* DNA methylation.

Selective DNA methylation defects in cellular gene sequences

The vast majority of the cellular DNA sequences that have been found to be aberrantly hypomethylated in ICF cells are repetitive elements of some kind [satellites 2 and 3, non-satellite repeats and parasitic elements (13,16,45)]. The functional transcribed genes most convincingly and consistently hypomethylated and aberrantly expressed in ICF syndrome cells appear to all reside on the inactive X chromosome in female ICF cells (18,27). We extended our search for DNA methylation defects to cellular genes. Technically, it is difficult to select a group of genes that is constitutively methylated in normal cells. We have taken advantage of a unique class of genes, termed cancer–testis (C–T) genes, most of which reside on the X chromosome. This class of genes is characterized by a highly restricted expression pattern (restricted to male germline cells) and promoters rich in CpG sites (CpG islands) (28,46). One of the most interesting features of the C–T genes examined in detail so far is that their CpG island promoters are always hypermethylated in normal somatic cells on one or both alleles, respectively, in male or female cells (for C–T genes on the X chromosome). Also, DNA methylation is thought to be the primary means of regulating their expression (28). BGS was utilized to examine the methylation status of two well-characterized C–T genes: *MAGE-A1* and *LAGE-1/2* (Fig. 2A). Results indicated that the

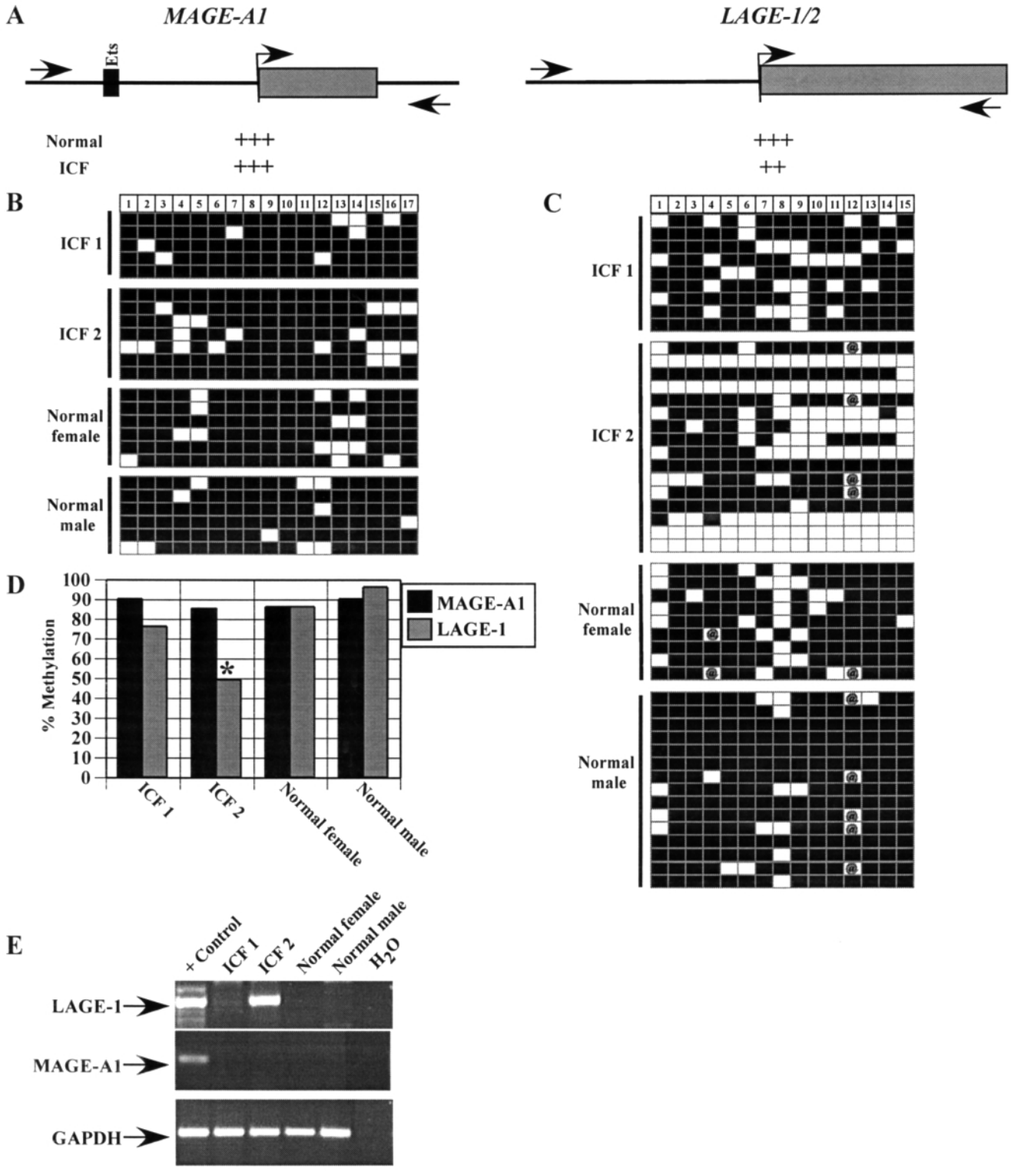

Figure 2. (**A**) Schematic of the *MAGE-A1* and *LAGE-1/2* cellular gene promoters examined in this study. A binding site for the Ets transcription factor, important for *MAGE-A1* activity, is also shown. (**B**) and (**C**) BGS analysis of *MAGE-A1* (B) and *LAGE-1/2* (**C**) in ICF and normal LCLs. Note that *LAGE-1* and *LAGE-2* could not be distinguished owing to the high degree of homology of these two genes as described previously (28). '@' represents CpG sites abolished by mutations. Numbers at the top of each table correspond to CpG sites within the region analyzed. (**D**) Quantitation of BGS data as the percentage methylation at all CpG sites for *MAGE-A1* (black) and *LAGE-1/2* (gray). The asterisk denotes the ICF sample showing hypomethylation of the *LAGE-1/2* promoter region. (**E**) Expression of *MAGE-A1* and *LAGE-1* by semiquantitative RT–PCR. GAPDH amplification served as a control for sample integrity. Amplifications were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining. Positive (+ control) and negative (H_2O) amplification controls are also shown.

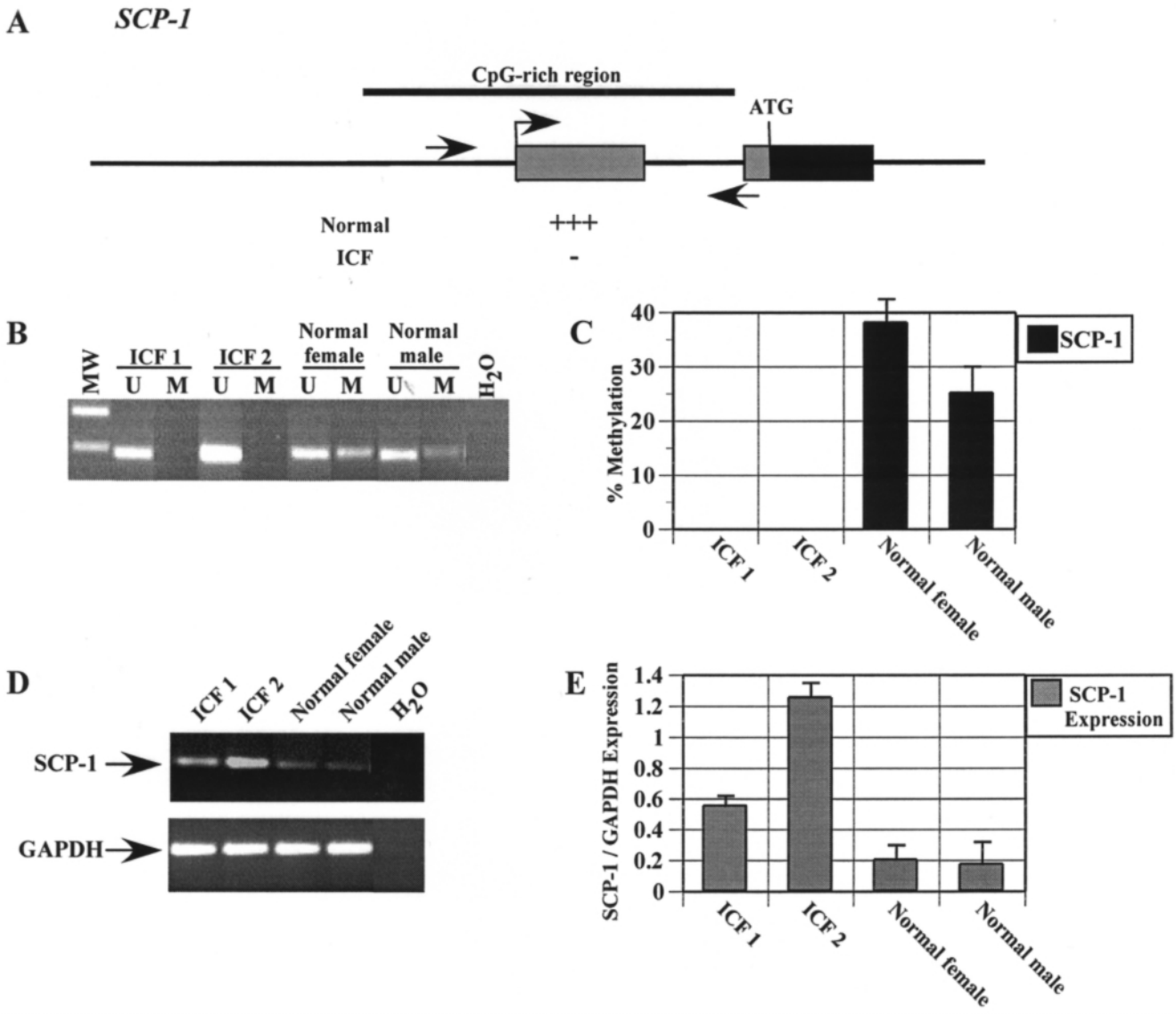

Figure 3. (**A**) Schematic of the *SCP-1* gene promoter (sequence derived from GenBank accession no. AL138783). The bent arrow represents the transcription start site; boxes are exons (black indicates the translated region of exon 2). (**B**) MSP methylation analysis of ICF and normal LCLs for SCP-1. (**C**) Quantitation of MSP data from (B) represented as the percentage methylation [M/(U + M) signals]. (**D**) SCP-1 expression monitored by semiquantitative RT–PCR. (**E**) Quantitation of SCP-1 mRNA levels relative to a control amplification for GAPDH mRNA. Amplifications were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining. A negative amplification control (H_2O) is also shown. Results of all PCR reactions are presented as the mean of triplicate experiments ± SD.

MAGE-A1 CpG island promoter was heavily methylated in all cell lines examined (Fig. 2B and D), and expression of the gene was not detectable by RT–PCR (Fig. 2E). This is consistent with previous studies (28). The *LAGE-1/2* CpG island promoter was heavily methylated in ICF 1 and the normal controls; however, we found a large degree of hypomethylation of this region in the ICF 2 cell line (Fig. 2C and D). We observed a ~2-fold decrease in methylation at the *LAGE-1/2* promoter for ICF 2 (Fig. 2D), and, interestingly, this was the only cell line positive for LAGE-1 expression by RT–PCR (Fig. 2E). A number of the clones sequenced from this region were completely unmethylated at all CpG sites. As further comparison, we examined the methylation status of the CpG island promoter for a gene known to become aberrantly hypermethylated in tumor cells, $p14^{ARF}$ (47,48). This gene resides on human chromosome 9. MSP revealed that there was no detectable methylation of this region in any of the cell lines, and RT–PCR analysis showed that the gene was equally expressed in all cell lines (data not shown).

The vast majority of C–T genes identified to date reside on the X chromosome, along with most of the genes shown to be hypomethylated in ICF cells. Thus, it may be that genes on the inactive X chromosome as a whole are more sensitive to the ICF mutation. Alternatively, C–T genes, because of their particular reliance on DNA methylation to maintain their highly restricted expression pattern, may be sensitive to the

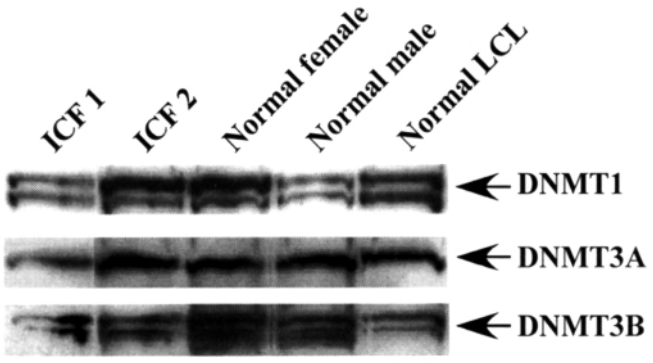

Figure 4. Western blot protein expression analysis of normal and ICF LCLs for each of the three known, catalytically active, DNA methyltransferases: DNMT1, DNMT3A and DNMT3B. Equal mass amounts of nuclear protein were loaded into each lane of a 6% SDS–PAGE gel, electrophoresed, transferred to PVDF membrane, and probed with antibodies specific for each DNMT. The same blot was stripped and reprobed with each of the antibodies shown. The DNMT3A and DNMT3B antibodies do not cross-react with each other.

DNMT3B mutations in ICF syndrome regardless of their chromosomal location. To examine this further, we determined the methylation and expression status of an autosomal C–T gene called synaptonemal, complex protein 1 (*SCP-1*), located on chromosome 1p13. SCP-1 is expressed only in the meiotic prophase of spermatocytes, and is involved in meiotic chromosome synapsis of sperm cells (49). The gene also contains a CpG island at its 5′ end (50). MSP analysis of the CpG-rich region of the *SCP-1* promoter (Fig. 3A) showed that it was markedly hypomethylated in both ICF 1 and ICF 2 compared with the normal controls (Fig. 3B and C). MSP also revealed a high degree of hypermethylation of the *SCP-1* CpG island in normal cells, which is consistent with other more well-characterized C–T genes (28). RT–PCR expression analysis showed that, unlike MAGE-A1 and LAGE-1/2, SCP-1 mRNA was detectable in both normal and ICF cells (Fig. 3D), consistent with the finding of unmethylated alleles in the normal control cell lines by MSP. However, a 3–6-fold increase in SCP-1 transcript levels was observed in the ICF cell lines by RT–PCR (Fig. 3E). The finding of low-level expression of SCP-1 in the normal controls may indicate that factors other than DNA methylation are also involved in regulating the expression of this gene in LCLs.

Expression levels of the three known catalytically active DNA methyltransferases (DNMTs) in ICF cells

Our methylation and expression analysis of EBV sequences revealed that there is a global defect in new *de novo* methylation in ICF cells. There was, however, some *de novo* methylation detectable (Fig. 1B and C). There are currently three known DNMTs that have been shown to be catalytically active *in vivo* and *in vitro* (1,30,51–53). We wondered if the levels of the other DNMTs (DNMT1 and 3A) might be aberrantly expressed in ICF cells in an attempt by the cell to compensate for the loss of functional DNMT3B. Protein levels of DNMT1, 3A and 3B were examined by western blot using nuclear extracts prepared from normal and ICF cell lines. Our results indicate that levels of DNMT1 were not grossly altered in the ICF cells relative to the normal controls (Fig. 4). The levels of DNMT3A were nearly identical in all cell lines. Western blotting for DNMT3B indicated that the mutant proteins were stable enough to be detected and that the normal female and male cell lines expressed mildly elevated levels of DNMT3B, although we do not know if this small but reproducible difference has functional significance. The two closely spaced bands in the DNMT1 and DNMT3B westerns are most likely post-translationally modified forms and splice variants, respectively, and can be observed with other antibodies as well (4, 52; and data not shown).

DISCUSSION

In the present work, we show for the first time that the *de novo* methylation of newly introduced sequences, known to undergo regulated *de novo* methylation events, is globally defective in ICF syndrome cells. Our data further underscore the notion that genes on the inactive X chromosome in female ICF cells are sensitive to the ICF *DNMT3B* mutations. Results also indicate that the C–T gene family may be a new class of DNA sequences that are also reliant on DNMT3B for their proper methylation, regardless of their chromosomal location. Aberrant hypomethylation was highly correlated with changes in expression of the associated genes.

EBV as a model system for studying *de novo* methylation

We made use of the well-characterized nature of EBV biology, in particular the defined *de novo* methylation events that the virus is known to undergo upon infection and establishment of an immortalized lymphoblastoid cell line, to gain insights into the nature of the methylation defect in ICF syndrome. The EBV virion DNA is hypomethylated (35), and provides both an efficient gene delivery system and a set of DNA sequences known to become *de novo* methylated as a part of their normal biology. This system also removes the complicating influence of genome-wide methylation remodeling events that occur during embryonic development and to which all cellular DNA sequences are subject (54). Our studies also reveal that ICF cells retain the capacity for some *de novo* methylation, since, although methylation levels were highly reduced in all regions of EBV that we examined, some methylation, especially in the Wp region, could still be detected. This is consistent with *Dnmt3a* and *Dnmt3b* mouse knockout studies, which indicated that while there was some functional redundancy between the *de novo* methyltransferases, the presence of one DNMT3 family member could not completely compensate for loss of the other (1). We were able to detect expression of all three known catalytically active DNA methyltransferases (DNMT1, 3A and 3B) in the ICF cell lines, and although there were small variations in the expression levels between normal and ICF cells for some of the DNMTs, no ICF-specific differences were observed (such as upregulation of DNMT3A). The fact that all EBV regions examined were highly hypomethylated in ICF cells, yet our analysis of the *MAGE/LAGE* cellular gene region indicated normal or near-normal levels of methylation [these regions are hypomethylated in sperm and so must be *de novo*

methylated during development (28)], reinforces the notion that ICF cells retain significant *de novo* methylation activity toward cellular gene sequences during the methylation remodeling phases of embryonic development.

One of the advantages of using the EBV system to monitor *de novo* methylation events is that it should provide a 'snapshot' of the capacity of primary ICF patient-derived B cells first put into culture to perform *de novo* methylation. This *de novo* methylation would then be copied by the maintenance DNA methyltransferase DNMT1 through subsequent cell divisions and the establishment of the immortalized cell line. It is known, however, that *in vitro* cell culture may lead to aberrant hypermethylation events, particularly in genes not necessary under cell culture conditions (55,56). While some of the methylation that we are observing may be due to cell culture or the immortalization process, the fact that we can still observe clear methylation differences after prolonged culture argues against the idea that we are measuring only cell culture- or immortalization-induced artifacts. It has been shown by others that methylation patterns within an EBV episome are stably maintained (57). Thus, although we were unable to perform these studies using primary ICF B cells owing to the rarity of ICF syndrome, it seems reasonable to conclude that the methylation defects found in the established cell lines would also be observed in a very early-passage culture of ICF B cells infected with EBV. An additional limitation to these studies is that we were restricted to using lymphocytes, since EBV is only capable of infecting and immortalizing B cells. The mechanisms regulating *de novo* methylation are still poorly understood at present; however, there is currently no evidence to suggest that the machinery involved in *de novo* methylation is different in B cells compared with other cell types. Another factor to be considered when using the EBV-based system is that the EBV genome is a viral sequence that is maintained as an autonomously replicating episome, which replicates once per division with the host genome. Numerous previous studies have shown, however, that EBV-based episomal systems are excellent models for studying various aspects of DNA methylation, including *de novo* methylation (57–59). The episome is replicated almost entirely by cellular factors during latency, and has been shown to be packaged into chromatin in a manner analogous to cellular genes (60). Thus we feel that these features make the EBV-based system an ideal model for studying *de novo* methylation and provide additional support that the results we have obtained using this system truly reflect the cellular phenotype of the ICF methylation defect. Lastly, it should be noted that while the only known genetic difference between the normal and ICF cells is the presence of a mutant *DNMT3B* gene, the methylation defects that we observed in both the viral and cellular sequences may be influenced by indirect effects of *DNMT3B* mutation. These could include changes in chromatin structure, alterations in protein binding, and aberrant expression of other genes involved in regulating DNA methylation patterns.

Methylation defects in cellular sequences compared with viral sequences

We extended our analysis of viral DNA sequences to a unique class of cellular genes: the cancer–testis (C–T) gene family. Members of this class of genes include the *MAGE* family (18 related genes), *GAGE* and *LAGE/ESO* families, all of which reside on the X chromosome (28). An autosomal C–T gene, *SCP-1*, has also been identified (49). The promoter regions of these genes are rich in CpG sites [and fit the definition of a CpG island as described in (61)] and (unlike most CpG islands) are hypermethylated, and the associated genes are silent, in all normal cells with the exception of male germline cells (28). DNA methylation appears to be the primary silencing mechanism for the *MAGE* and *LAGE* genes, and therefore we thought that they would be good candidates for analysis in ICF cells. BGS and RT–PCR analysis showed that the heavily methylated status of *MAGE-A1* and *LAGE-1* was largely maintained in ICF cells. Previous studies have indicated that genes on the inactive X chromosome, unlike autosomal genes, may be sensitive to the ICF mutation (18). We observed a ~50% decrease in methylation of the *LAGE-1* promoter in the ICF 2 cell line. Given that ICF 1 is derived from a male ICF patient and ICF 2 is derived from a female ICF patient, it is likely that we are observing loss of methylation and re-expression from a single allele of *LAGE-1* in ICF 2, especially since many of the sequenced clones were hypomethylated at all CpG sites in an individual molecule. Thus our results support the notion that some, but not all, genes on the inactive X chromosome may be reliant on DNMT3B for proper methylation and gene silencing. Our data from the *SCP-1* C–T gene on chromosome 1p13 shows that, while its expression did not appear to be as tightly regulated as for the *MAGE/LAGE* genes, its methylation was markedly reduced in the ICF cells. Thus, the C–T gene family may represent a previously unrecognized class of genes that are reliant on DNMTB for proper *de novo* methylation. Owing to the low occurrence of ICF syndrome, our studies were limited to only two ICF cell lines. Future work will include analysis of additional C–T genes in these and other ICF cell lines as they become available. The comparison between viral and cellular genes also underscores the additional levels of complexity in understanding the ICF phenotype when studying only cellular genes, which have been exposed to complex and poorly understood demethylation and remethylation events occurring during embryonic development and differentiation (54).

Could the defects in *de novo* methylation that we observed be in some way related to an intrinsic property of the DNA sequences themselves, or do other factors play a role? Analysis of the sequences examined by BGS and MSP, in terms of GC content and frequency of the CpG dinucleotide, indicates that the *MAGE/LAGE/SCP-1* gene regions are ~2–3-fold more CpG-rich than the EBV promoter regions examined. However, we observed that the Zp region was never hypermethylated and that the *MAGE-A1* region was faithfully hypermethylated in ICF cells; thus, the intrinsic composition of the DNA sequence does not appear to play a consistent role. Does the specific *DNMT3B* mutation in each of the ICF cells play a role in the severity of the defects that we observed? As mentioned previously, the ICF 1 cell line contains a homozygous V726G mutation in *DNMT3B* and the ICF 2 cell line is heterozygous for an A603T mutation and an intron 22 G-to-A mutation resulting in a three-amino-acid insertion (STP) in DNMT3B (1,3,12). Given that ICF 1 and ICF 2 are derived from patients of different sex, we cannot

draw conclusions on the effects of the *DNMT3B* mutations using the X-linked genes. Considering the EBV sequences and the *SCP-1* gene, however, indicates that the heterozygous mutations in *DNMT3B* in ICF 2 may be more severe (consistently lower methylation in Cp and Wp regions and higher expression for SCP-1). The V726G mutation occurs between conserved methylase motifs VI and IX, a region thought to be involved in DNA binding, while the A603T mutation occurs near conserved methylase motif I, thought to be involved in cofactor (*S*-adenosyl-L-methionine) binding; and the three-amino-acid insertion (STP) also occurs between conserved methylase motifs VI and IX, and could therefore have an impact on DNA binding (62). The DNMT3B proteins present in the cells of ICF 2 may be functionally impaired to a greater degree owing to a reduced ability to bind DNA compared with the homozygous mutation in DNMT3B in ICF 1 cells. Additional structure–function studies and examination of significantly more ICF cell lines, which are quite rare, will be required before any firm correlation can be made.

Our results clearly show that ICF-specific mutations in *DNMT3B* result in impaired *de novo* methylation of newly introduced DNA sequences known to normally undergo *de novo* methylation. We also show additional evidence that specific genes on the X chromosome are susceptible to mutations in *DNMT3B* and that the levels of other DNMTs are not consistently altered in ICF cells. Our analysis also indicates that the C–T gene family may represent a new class of sequences sensitive to *DNMT3B* mutations, regardless of their chromosomal location. Furthermore, our results indicate that *de novo* methylation is not completely eliminated by mutations in *DNMT3B*, and therefore either these mutations do not completely eliminate DNMT3B catalytic activity or other DNMTs, such as DNMT3A, are also involved in *de novo* methylation. However, the other DNMTs still cannot compensate for the methylation functions of DNMT3B. Lastly, our data illustrate the utility of the EBV-based system for dissecting the complex and poorly understood process of *de novo* methylation, and future studies with this system should continue to provide useful insights into how mutations in *DNMT3B* affect DNA methylation.

MATERIALS AND METHODS

Cell lines and tissue culture

EBV-immortalized LCLs used in these studies were derived from two ICF patients: patient B from (8) and patient C [GM 08714, NIGMS Human Mutant Cell Repository (31)]. These patients are referred to as 'ICF 1' and 'ICF 2', respectively, in this paper. Patient B is a male homozygous for a V726G mutation (3) and patient C is a female heterozygous for an A603T mutation and an intron 22 G-to-A mutation resulting in a three-amino-acid (STP) insertion (1). Normal control LCLs included patient C's parents [GM 08728 (mother) and GM 08729 (father), referred to here as 'normal female' and 'normal male'] and another unrelated LCL ['normal LCL'] purchased from the American Type Culture Collection (CCL 256.1). Cells were maintained in RPMI1640 supplemented with 15% heat-inactivated fetal calf serum (Life Technologies).

Table 1. Bisulfite genomic sequencing (BGS), MSP and RT–PCR primers used in this study

Promoter	Primer name	Sequence (5′→3′)
Cp (MSP), methylated	Sense	GTCCGAAAACCGAACGACTCG
	Antisense	GTAAGGCGTAATTAATTTCGTTC
Cp (MSP), unmethylated	Sense	CATCCAAAAACCAAACAACTCA
	Antisense	AGTAAGGTGTAATTAATTTTGTTT
Wp (MSP), methylated	Sense	CGTTATTTTGTTTTTACGCGC
	Antisense	AAAAAAATAACCTAACAACGCG
Wp (MSP), unmethylated	Sense	GTGTTATTTTGTTTTTATGTGT
	Antisense	CTATCTAACTTACATAAACACA
Zp (BGS)	Sense	AAAAAACACCTAATATAAATCAAA
	Antisense	GTTAAAGAGGATTAGGTTTTTTT
Rp (BGS)	Sense	TCTTTTATAAACCATTAACATAAA
	Antisense	GAGATTGATAGGATTATATTGT
MAGE-A1 (BGS)	Sense	TTTTATTTTTATTTAGGTAGGATT
	Antisense	CTAATATCTCTCAAAACTTTTAA
LAGE-1/2 (BGS)	Sense	GGGGTTTATTGGGGTTTTTTA
	Antisense	TACCAAAACCTCCTAAACCAT
SCP-1 (MSP), methylated	Sense	GTCGTTGAATTGTTAGTCGTTC
	Antisense	AAAAACAACGAAAAACTACGAC
SCP-1 (MSP), unmethylated	Sense	TAGTTGTTGAATTGTTAGTTGTTT
	Antisense	AAAAAAACAACAAAAAACTACAAC
Cp/Wp (RT–PCR)	Sense	TGGCGTGTGACGTGGTGTAA
	Antisense	CATTTCCAGGTCCTGTACCT
Rp (RT–PCR)	Sense	CAAACAGACGCAGATGAGGC
	Antisense	GCGGTGCCTATGGTGGCAGG
Zp (RT–PCR)	Sense	GGGAGAAGCACCTCAACCTG
	Antisense	TTGCTTAAACTTGGCCCGGC
MAGE-A1 (RT–PCR)	Sense	TGTGGGCAGGAGCTGGGCAA
	Antisense	GCCGAAGGAACCTGACCCAG
LAGE-1 (RT–PCR)	Sense	GCAGGATGGAAGGTGCCC
	Antisense	CTGGCCACTCGTGCTGGGA
SCP-1 (RT–PCR)	Sense	GAAGGAAACGAGGGTTTATTCC
	Antisense	CAAAGGGCTTTTGCTTTTCCAT
GAPDH (RT–PCR)	Sense	ATCTCTGCCCCCTCTGCTGA
	Antisense	GGATGACCTTGCCCACAGGGT

Bisulfite genomic sequencing (BGS)

Genomic DNA was extracted from fresh cell pellets with Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH). Two to five micrograms of genomic DNA was digested with *Eco*RI in a 30 µl volume, then denatured by adding 3 µl of 3 M NaOH at 37°C for 15 min. Denatured DNA was mixed with 333 µl of bisulfite solution and treated in darkness for 4 h at 55°C. The bisulfite solution was prepared as 2.4 M sodium metabisulfite (pH 5.0–5.2) (Sigma)/0.5 mM hydroquinone. Treated DNA was desalted and purified using the Qiaex II kit (Qiagen). DNA was then treated with 0.3 M NaOH at 37°C for 15 min and precipitated with 3 M ammonium acetate and 3 volumes of ethanol. Recovered DNA was dissolved in 20–50 µl of TE buffer (pH 8.0) and stored at −20°C. The bisulfite-treated DNA was PCR-amplified with strand-specific primers as described in (43,63). The PCR products were electrophoresed and purified using Spin-X tubes (Costar, Corning, NY), and then cloned into the pCR2.1–TOPO cloning vector (Invitrogen, Carlsbad, CA). Plasmid DNA was extracted and sequenced using the ABI PRISM BigDye

Terminator Cycle Sequencing Kit and a PE ABI 377 sequencer. Methylation-specific PCR (MSP) was performed exactly as described previously (26). Standardization curves of PCR cycle number versus product yield for MSP reaction were performed (data not shown), and the cycle numbers utilized were within the linear amplification range. All reactions were performed in triplicate. The primers used to amplify regions for BGS and MSP analysis are listed in Table 1. The BGS primers for *MAGE-A1* amplified the region between −105 and +225 and the *LAGE-1/2* primers amplified the region between −112 to +134, relative to the respective transcription initiation sites.

Semiquantitative RT–PCR

Total RNA was extracted from fresh cells using RNazol B (Teltest, Inc.). RT–PCR was performed as described in (63), using AmpliTaq Gold (PE Biosystem, Foster City, CA). The PCR program started with an initial denaturation at 95°C for 10 min, followed by 35–37 cycles (94°C for 30 s, 51°C or 60°C for 30 s, and 72°C for 30 s) of amplification, with a final extension at 72°C for 10 min. PCR bands were visualized under ultraviolet light, photographed, and quantitated with the EagleEye digital gel documentation system and the supplied densitometry software (EagleSight Software, version 3.2, Stratagene, La Jolla, CA). Initial studies on the standardization curves of RT–PCR cycle number versus product yield for the EBV and cellular genes showed that linear amplification occurred between 25 and 40 cycles (data not shown). Therefore 35–37 cycles of PCR were used for our semiquantitative RT–PCR analysis. All reactions were performed in triplicate. *GAPDH* was used as a control for RNA integrity, and only 20 cycles of amplification were used. The *GAPDH* primers were designed according to the GAPDH mRNA sequence (GenBank accession no. M33197). All primers used to amplify transcripts are listed in Table 1.

Nuclear extracts and western blotting

Nuclear extracts were prepared from normal and ICF cell lines exactly as described in (64). Western blotting was performed as described in (65), with rabbit antibodies raised against bacterially expressed recombinant protein (amino acids 914–1087, Covance) for DNMT1, or anti-peptide antibodies raised against DNMT3A (Santa Cruz P-16) and DNMT3B (peptide sequence ESPPPKRLKTNSYGGKDRGE, Research Genetics). The antibodies for DNMT3A and DNMT3B did not cross-react with each other (data not shown). For western blots, equal microgram amounts of nuclear protein were loaded into each lane, run on a 6% SDS–PAGE gel, and transferred to PVDF membrane for western blotting.

ACKNOWLEDGEMENTS

We thank Dr D. Smeets for providing the ICF 1 cell line. T.M.G was supported by a Pharmacology Research Associate Training (PRAT) fellowship, NIGMS, NIH. This work was supported by Grant CA84535-01 from the National Cancer Institute (K.D.R) and an NSTB research grant from Johns Hopkins Singapore (Q.T). K.D.R is a National Cancer Institute Scholar.

REFERENCES

1. Okano, M., Bell, D.W., Haber, D.A. and Li, E. (1999) DNA methyltransferases *Dnmt3a* and *Dnmt3b* are essential for *de novo* methylation and mammalian development. *Cell*, **99**, 247–257.
2. Xu, G.-L., Bestor, T.H., Bourc'his, D., Hsieh, C.-L., Tommerup, N., Bugge, M., Hulten, M., Qu, Russo, J.J. and Viegas-Pequignot, E. (1999) Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. *Nature*, **402**, 187–191.
3. Hansen, R.S., Wijmenga, C., Luo, P., Stanek, A.M., Canfield, T.K., Weemaes, C.M.R. and Gartler, S.M. (1999) The *DNMT3B* DNA methyltransferase gene is mutated in the ICF immunodeficiency syndrome. *Proc. Natl Acad. Sci. USA*, **96**, 14412–14417.
4. Robertson, K.D., Uzvolgyi, E., Liang, G., Talmadge, C., Sumegi, J., Gonzales, F.A. and Jones, P.A. (1999) The human DNA methyltransferases (DNMTs) 1, 3a, and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors. *Nucleic Acids Res.*, **27**, 2291–2298.
5. Xie, S., Wang, Z., Okano, M., Nogami, M., Li, Y., He, W.-W., Okumura, K. and Li, E. (1999) Cloning, expression and chromosome locations of the human DNMT3 gene family. *Gene*, **236**, 87–95.
6. Wijmenga, C., van den Heuvel, L.P.W.J., Strengman, E., Luyten, J.A.F.M., van der Burgt, I.J.A.M., de Groot, R., Smeets, D.F.C.M., Draaisma, J.M.T., van Dongen, J.J., De Abreu, R.A. *et al.* (1998) Localization of the ICF syndrome to chromosome 20 by homozygosity mapping. *Am. J. Hum. Genet.*, **63**, 803–809.
7. Robertson, K.D. (2001) DNA methylation, methyltransferases, and cancer. *Oncogene*, **20**, 3139–3155.
8. Smeets, D.F.C.M., Moog, U., Weemaes, C.M.R., Vaes-Peeters, G., Merkx, G.F.M., Niehof, J.P. and Hamers, G. (1994) ICF syndrome: a new case and review of the literature. *Hum. Genet.*, **94**, 240–246.
9. Franceschini, P., Martino, S., Ciocchini, M., Ciuti, E., Vardeu, M.P., Guala, A., Signorile, F., Camerano, P., Franceschini, D. and Tovo, P.A. (1995) Variability of clinical and immunological phenotype in immunodeficiency-centromeric instability-facial anamolies syndrome. *Eur. J. Pediatr.*, **154**, 840–846.
10. Narayan, A., Tuck-Muller, C., Weissbecker, K., Smeets, D. and Ehrlich, M. (2000) Hypersensitivity to radiation-induced non-apoptotic and apoptotic death in cell lines from patients with the ICF chromosome instability syndrome. *Mutat. Res.*, **456**, 1–15.
11. Ehrlich, M., Buchanan, K.L., Tsein, F., Jiang, G., Sun, B., Uicker, W., Weemaes, C.M.R., Smeets, D., Sperling, K., Belohradsky, B.H. *et al.* (2001) DNA methyltransferase 3B mutations linked to the ICF syndrome cause dysregulation of lymphomagenesis genes. *Hum. Mol. Genet.*, **10**, 2917–2931.
12. Tuck-Muller, C.M., Narayan, A., Tsien, F., Smeets, D.F.C.M., Sawyer, J., Fiala, E.S., Sohn, O.S. and Ehrlich, M. (2000) DNA hypomethylation and unusual chromosome instability in cell lines from ICF patients. *Cytogenet. Cell Genet.*, **89**, 121–128.
13. Jeanpierre, M., Turleau, C., Aurias, A., Prieur, M., Ledeist, F., Fischer, A. and Viegas-Pequignot, E. (1993) An embryonic-like methylation pattern of classical satellite DNA is observed in ICF syndrome. *Hum. Mol. Genet.*, **2**, 731–735.
14. Tagarro, I., Fernandez-Peralta, A.M. and Gonzales-Aguilera, J.J. (1994) Chromosomal localization of human satellites 2 and 3 by FISH method using oligonucleotides as probes. *Hum. Genet.*, **93**, 383–388.
15. Hassan, K.M.A., Norwood, T., Gimelli, G., Gartler, S.M. and Hansen, R.S. (2001) Satellite 2 methylation patterns in normal and ICF syndrome cells and association of hypomethylation with advanced replication. *Hum. Genet.*, **109**, 452–462.
16. Kondo, T., Bobek, M.P., Kuick, R., Lamb, B., Zhu, X., Narayan, A., Bourc'his, D., Viegas-Pequignot, E., Ehrlich, M. and Hanash, S.M. (2000) Whole-genome methylation scan in ICF syndrome: hypomethylation of non-satellite DNA repeats *D4Z4* and *NBL2*. *Hum. Mol. Genet.*, **9**, 597–604.
17. Schuffenhauer, S., Bartsch, O., Stumm, M., Buchholz, T., Petropoulou, T., Kraft, S., Belohradsky, B., Hinkel, G.K., Meitinger, T. and Wegner, R.-D. (1995) DNA, FISH and complementation studies in ICF syndrome: DNA hypomethylation of repetitive and single copy loci and evidence for a trans acting factor. *Hum. Genet.*, **96**, 562–571.
18. Hansen, R.S., Stoger, R., Wijmenga, C., Stanek, A.M., Canfield, T.K., Luo, P., Matarazzo, M.R., D' Esposito, M., Feil, R., Gimelli, G. *et al.* (2000) Escape from gene silencing in ICF syndrome: evidence for advanced replication time as a major determinant. *Hum. Mol. Genet.*, **9**, 2575–2587.

19. Frommer, M., McDonald, L.E., Millar, D.S., Collis, C.M., Watt, F., Grigg, G.W., Molloy, P.L. and Paul, C.L. (1992) A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc. Natl Acad. Sci. USA*, **89**, 1827–1831.
20. Liebowitz, D. and Kieff, E. (1993) Epstein–Barr virus. In Roizman, B., Whitley, R.J. and Lopez, C. (eds), *The Human Herpesviruses.* Raven Press, New York, pp. 107–172.
21. Woisetschlaeger, M., Yandava, C.N., Furmanski, L.A., Strominger, J.L. and Speck, S.H. (1990) Promoter switching in Epstein–Barr virus during the initial stages of infection of B lymphocytes. *Proc. Natl Acad. Sci. USA*, **87**, 1725–1729.
22. Tierney, R.J., Steven, N., Young, L.S. and Rickinson, A.B. (1994) Epstein–Barr virus latency in blood mononuclear cells: analysis of viral gene transcription during primary infection and in the carrier state. *J. Virol.*, **68**, 7374–7385.
23. Kinter, C. and Sugden, B. (1981) Conservation and progressive methylation of Epstein-Barr viral DNA sequences in transformed cells. *J. Virol.*, **38**, 305–316.
24. Robertson, K.D. (2000) The role of DNA methylation in modulating Epstein–Barr virus gene expression. In Vogt, P. and Jones, P.A. (eds), *Curr. Top. Microbiol. Immunol., DNA Methylation and Cancer.* Springer-Verlag, Berlin, Vol. **249**, pp. 21–34.
25. Tierney, R.J., Kirby, H.E., Nagra, J.K., Desmond, H., Bell, A.I. and Rickinson, A.B. (2000) Methylation of transcription factor binding sites in the Epstein–Barr virus latent cycle promoter Wp coincides with promoter down-regulation during virus-induced B-cell transformation. *J. Virol.*, **74**, 10468–10479.
26. Tao, Q., Swinnen, L.J., Yang, J., Srivastava, G., Robertson, K.D. and Ambinder, R.F. (1999) Methylation status of the Epstein–Barr virus major latent promoter C in iatrogenic B-cell lymproliferative disease: application of PCR-based analysis. *Am. J. Pathol.*, **155**, 619–625.
27. Miniou, P., Jeanpierre, M., Blanquet, V., Sibella, V., Bonneau, D., Herbelin, C., Fischer, A., Niveleau, A. and Viegas-Pequignot, E. (1994) Abnormal methylation pattern in constitutive and facultative (X inactive chromosome) heterochromatin of ICF patients. *Hum. Mol. Genet.*, **3**, 2093–2102.
28. De Smet, C., Lurquin, C., Lethe, B., Martelange, V. and Boon, T. (1999) DNA methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG-rich promoter. *Mol. Cell. Biol.*, **19**, 7327–7335.
29. Lin, I.G., Han, L., Taghva, A., O'Brien, L.E. and Hsieh, C.-L. (2002) Murine *de novo* methyltransferase Dnmt3a demonstrates strand assymetry and site preference in the methylation of DNA *in vitro*. *Mol. Cell. Biol.*, **22**, 704–723.
30. Yokochi, T. and Robertson, K.D. (2002) Preferential methylation of unmethylated DNA by mammalian *de novo* DNA methyltransferase Dnmt3a. *J. Biol. Chem.*, **277**, 11735–11745.
31. Carpenter, N.J., Fillpovich, A., Blaese, M., Carey, T.L. and Berkel, I. (1988) Variable immunodeficiency with abnormal condensation of the heterochromatin of chromosomes 1, 9, and 16. *J. Pediat.*, **112**, 757–760.
32. Herman, J.G., Graff, J.R., Myohanen, S., Nelkin, B.D. and Baylin, S.B. (1996) Methylation-specific PCR: a novel assay for methylation status of CpG islands. *Proc. Natl Acad. Sci. USA*, **93**, 9821–9826.
33. Larocca, D., Homisak, D. and Clough, W. (1981) Synthesis of hypomethylated Epstein–Barr viral DNA is stimulated by dimethylsulfoxide treatment of lymphoblastoid cells. *Biochem. Biophys. Res. Commun.*, **100**, 559–565.
34. Perlmann, C., Saemundsen, A.K. and Klein, G. (1982) A fraction of Epstein–Barr virus virion DNA is methylated in and around the *Eco*RI-J fragment. *Virology*, **123**, 217–221.
35. Szyf, M., Eliasson, L., Mann, V., Klein, G. and Razin, A. (1985) Cellular and viral DNA hypomethylation associated with induction of Epstein–Barr virus lytic cycle. *Proc. Natl Acad. Sci. USA*, **82**, 8090–8094.
36. Woisetschlaeger, M., Jin, X.W., Yandava, C.N., Furmanski, L.A., Strominger, J.L. and Speck, S.H. (1991) Role for the Epstein–Barr virus nuclear antigen 2 in viral promoter switching during initial stages of infection. *Proc. Natl Acad. Sci. USA*, **88**, 3942–3946.
37. Ling, P.D., Rawlins, D.R. and Hayward, S.D. (1993) The EBV immortalizing protein EBNA-2 is targeted to DNA by a cellular enhancer-binding protein. *Proc. Natl Acad. Sci. USA*, **90**, 9237–9241.
38. Manet, E., Gruffat, H., Trescol-Biemont, M.C., Moreno, N., Chambard, P., Giot, J.F. and Seargent, A. (1989) Epstein-Barr virus bisistronic mRNAs generated by facultative splicing code for two transcriptional *trans*-activators. *EMBO J.*, **8**, 1819–1826.
39. Lieberman, P.M., Hardwick, J.M., Sample, J., Hayward, G.S. and Hayward, S.D. (1990) The Zta transactivator involved in induction of lytic cycle gene expression in Epstein–Barr virus-infected lymphocytes binds to both AP-1 and ZRE sites in target promoter and enhancer regions. *J. Virol.*, **64**, 1143–1155.
40. Sinclair, A.J., Brimmell, M., Shanahan, F. and Farrell, P.J. (1991) Pathways of activation of the Epstein–Barr virus productive cycle. *J. Virol.*, **65**, 2237–2244.
41. Bogedain, C., Alliger, P., Schwarzmann, F., Marschall, M., Wolf, H. and Jilg, W. (1994) Different activation of Epstein–Barr virus immediate–early and early genes in Burkitt lymphoma cells and lymphoblastoid cell lines. *J. Virol.*, **68**, 1200–1203.
42. Zalani, S., Holley-Guthrie, E. and Kenney, S. (1996) Epstein–Barr viral latency is disrupted by the immediate–early BRLF1 protein through a cell-specific mechanism. *Proc. Natl Acad. Sci. USA*, **93**, 9194–9199.
43. Robertson, K.D., Manns, A., Swinnen, L.J., Zong, J.C., Gulley, M.L. and Ambinder, R.F. (1996) CpG methylation of the major Epstein–Barr virus latency promoter in Burkitt's lymphoma and Hodgkin's disease. *Blood*, **88**, 3129–3136.
44. Robertson, K.D. and Ambinder, R.F. (1997) Methylation of the Epstein–Barr virus genome in normal lymphocytes. *Blood*, **90**, 4480–4484.
45. Miniou, P., Bourc'his, D., Gomes, D.M., Jeanpierre, M. and Viegas-Pequignot, E. (1997) Undermethylation of Alu sequences in ICF syndrome: molecular and *in situ* analysis. *Cytogenet. Cell Genet.*, **77**, 308–313.
46. Old, L.J. and Chen, Y.-T. (1998) New paths in human cancer serology. *J. Exp. Med.*, **187**, 1163–1167.
47. Robertson, K.D. and Jones, P.A. (1998) The human ARF cell cycle regulatory gene promoter is a CpG island which can be silenced by DNA methylation and down-regulated by wild-type p53. *Mol. Cell. Biol.*, **18**, 6457–6473.
48. Esteller, M., Tortola, S., Capella, G., Peinado, M.A., Baylin, S.B. and Herman, J.G. (2000) Hypermethylation-associated inactivation of p14 (ARF) is independent of p16(INK4a) methylation and p53 mutational status. *Cancer Res.*, **60**, 129–133.
49. Tureci, O., Sahin, U., Zwick, C., Koslowski, M., Seitz, G. and Pfreundschuh, M. (1998) Identification of a meiosis-specific protein as a member of the cancer/testis antigens. *Proc. Natl Acad. Sci. USA*, **95**, 5211–5216.
50. Cross, S.H., Charlton, J.A., Nan, X. and Bird, A.P. (1994) Purification of CpG islands using a methylated DNA binding column. *Nat. Genet.*, **6**, 236–244.
51. Li, E., Bestor, T.H. and Jaenisch, R. (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell*, **69**, 915–926.
52. Okano, M., Xie, S. and Li, E. (1998) Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nat. Genet.*, **19**, 219–220.
53. Aoki, A., Suetake, I., Miyagawa, J., Fujio, T., Chijiwa, T., Sasaki, H. and Tajima, S. (2001) Enzymatic properties of *de novo*-type mouse DNA (cytosine-5) methyltransferases. *Nucleic Acids Res.*, **29**, 3506–3512.
54. Reik, W., Dean, W. and Walter, J. (2001) Epigenetic reprogramming in mammalian development. *Science*, **293**, 1089–1093.
55. Antequera, F., Boyes, J. and Bird, A. (1990) High levels of *de novo* methylation and altered chromatin structure at CpG islands in cell lines. *Cell*, **62**, 503–514.
56. Jones, P.A., Wolkowicz, M.J., Rideout, W.M., Gonzales, F.A., Marziasz, C.M., Coetzee, G. and Tapscott, S.J. (1990) *De novo* methylation of the *MyoD1* CpG island during the establishment of immortal cell lines. *Proc. Natl Acad. Sci. USA*, **87**, 6117–6121.
57. Hsieh, C.-L. (1997) Stability of patch methylation and its impact in regions of transcriptional initiation and elongation. *Mol. Cell. Biol.*, **17**, 5897–5904.
58. Hsieh, C.-L. (1999) Evidence that protein binding specifies sites of DNA demethylation. *Mol. Cell. Biol.*, **19**, 46–56.
59. Hsieh, C.-L. (1999) *In vivo* activity of murine *de novo* methyltransferases, Dnmt3a and Dnmt3b. *Mol. Cell. Biol.*, **19**, 8211–8218.
60. Shaw, J.E., Levinger, L.F. and Carter, C.W. (1979) Nucleosomal structure of Epstein–Barr virus DNA in transformed cell lines. *J. Virol.*, **29**, 657–665.

61. Gardiner-Garden, M. and Frommer, M. (1987) CpG islands in vertebrate genomes. *J. Mol. Biol*, **196**, 261–282.
62. Kumar, S., Cheng, X., Klimasauskas, S., Mi, S., Posfai, J., Roberts, R.J. and Wilson, G.G. (1994) The DNA (cytosine-5) methyltransferases. *Nucleic Acids Res.*, **22**, 1–10.
63. Tao, Q., Robertson, K.D., Manns, A., Hildesheim, A. and Ambinder, R.F. (1998) Epstein–Barr virus (EBV) in endemic Burkitt's lymphoma: molecular analysis of primary tumor tissue. *Blood*, **91**, 1373–1381.
64. Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.*, **11**, 1475–1489.
65. Robertson, K.D., Ait-Si-Ali, S., Yokochi, T., Wade, P.A., Jones, P.L. and Wolffe, A.P. (2000) DNMT1 forms a complex with Rb, E2F1, and HDAC1 and represses transcription from E2F-responsive promoters. *Nat. Genet.*, **25**, 338–342.