Hypomethylation of subtelomeric regions in ICF syndrome is associated with abnormally short telomeres and enhanced transcription from telomeric regions

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Telomeres and adjacent subtelomeric regions are packaged as heterochromatin in many organisms. The heterochromatic features include DNA methylation, histones H3-Lys9 (Lysine 9) and H4-Lys20 (Lysine 20) methylation and heterochromatin protein1 alpha binding. Subtelomeric DNA is hypomethylated in human sperm and ova, and these regions are subjected to de novo methylation during development. In mice this activity is carried out by DNA methyltransferase 3b (Dnmt3b). Mutations in DNMT3B in humans lead to the autosomal-recessive ICF (immunodeficiency, centromeric region instability, facial anomalies) syndrome. Here we show that, in addition to several satellite and non-satellite repeats, the subtelomeric regions in lymphoblastoid and fibroblast cells of ICF patients are also hypomethylated to similar levels as in sperm. Furthermore, the telomeres are abnormally short in both the telomerase-positive and -negative cells, and many chromosome ends lack detectable telomere fluorescence in situ hybridization signals from either one or both sister-chromatids. In contrast to Dnmt3a/b²/² mouse embryonic stem cells, increased telomere sister-chromatid exchange was not observed in ICF cells. Hypomethylation of subtelomeric regions was associated in the ICF cells with advanced telomere replication timing and elevated levels of transcripts emanating from telomeric regions, known as TERRA (telomeric-repeat-containing RNA) or TelRNA. The current findings provide a mechanistic explanation for the abnormal telomeric phenotype observed in ICF syndrome and highlights the link between TERRA/TelRNA and structural telomeric integrity.

INTRODUCTION

Telomeres are specialized nucleoprotein complexes that maintain the integrity and stability of eukaryotic chromosome ends (1–3). In mammalian cells, the telomeres and adjacent subtelomeric regions are packaged as constitutive heterochromatin, facilitating the stabilization and capping of chromosomes ends (4). The histone modifications in telomeric regions include enrichment of tri-methylation of histone H3 at Lysine 9 at Lysine 20 and low levels of acetylation of histones H3 and H4. In addition, heterochromatin protein1 alpha binds these regions, and subtelomeric-DNA is heavily methylated (reviewed in 5,6). Most recently, it has been shown that although devoid of genes, the subtelomeric and telomeric regions have the potential to transcribe telomeric-repeat-containing RNA (TERRA), also designated Telomeric RNA (TelRNA) (7,8).

While the dinucleotide CG is absent from the mammalian telomeric TTAGGG repeat, human subtelomeric regions are rich in CG sites (9–11). Human subtelomeric regions are hypomethylated in sperm and oocytes and are methylated de novo during development, resulting in heavy methylation of these regions in somatic cells (9–11). Other repetitive sequences such as human satellite 2 and NBL2 (12–16) and mouse pericentromeric satellite DNA (major and minor satellites) (17–19) undergo similar changes in DNA methylation during

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development. The DNA methyltransferases (DNMTs) 3A and 3B are the enzymes responsible for de novo DNA methylation in mammalian cells, with DNMT3B being the major enzyme involved in the de novo methylation of repetitive sequences (20–24). In mouse embryonic stem cells (mES) null for Dnmt3a and Dnmt3b (Dnmt3a/b−/−) subtelomeric regions, as well as major satellite sequences, are hypomethylated, but no abnormality was found with respect to histone modifications in these regions (25). The telomeres in Dnmt3a/b−/− mES cells are abnormally long, a phenotype that has been attributed to elevated sister-chromatid exchange at subtelomeric regions due to the hypomethylated state (25).

In humans, mutations in the DNMT3B gene (26–31) result in a rare autosomal-recessive inherited disease named ICF (immunodeficiency, centromeric instability, facial anomalies) type I syndrome (reviewed in 26,32,33). In ICF type I individuals, several repetitive sequences such as satellites 2 and 3, as well as the non-satellite NBL2 and D4Z4 repeats, are hypomethylated (12,13,15,16,29,31). In addition, several gene-associated CpG islands and LINE-1 repeats were found to be hypomethylated in the inactive X-chromosome of some of the female ICF patients (34–37). In association with the disturbance in the methylation status, the replication timing of the hypomethylated satellite 2 repeats and of several CpG islands on the inactive X-chromosome is slightly advanced in S-phase (35,38, and reviewed in 39). Histone modifications, on the other hand, show a normal pattern on the inactive X-chromosome of female ICF patients (40).

The main cytogenetic abnormality in the ICF patients is decondensation of satellite 2 regions which are localized at the juxtacentromeric heterochromatin regions of chromosomes 1 and 16, and more rarely of satellite 3 that is located on chromosome 9 (41). Occasionally these decondensed regions recombine and form multiradial chromosomes leading to prominent chromosomal instability (12,16,31,42, reviewed in 43). Anaphase bridges also occur at elevated rates in ICF cells (42). These have been attributed both to the centromeric abnormalities and to random telomeric associations between chromosomes (16,42). Abnormally short and/or unprotected telomeres are a major cause for telomeric fusions and chromosomal instability (44,45), and indeed fluorescence in situ hybridization (FISH) to telomeric regions indicated an elevated rate of telomere associations (46). In addition, 5-azacytidine-induced hypomethylation in normal PHA-activated T-lymphocytes is also associated with an elevated frequency of telomere associations (46).

In view of the discordant findings regarding the telomere length phenotype associated with mutations in DNMT3B (25,42), and the unclear relationship between telomere length and telomerase activity when such mutations are present, we have studied the subtelomeric methylation status and telomere length in three ICF patients, including both telomerase positive and negative cells. In addition, we have studied the effect of hypomethylation on the replication timing of these regions, which in turn may influence the transcriptional capacity of the region. Finally, we have studied the transcription of TERRA/TelRNA in the ICF cells in comparison with normal controls. Based on these findings, we suggest a possible mechanism relating the hypomethylated state of the subtelomeric regions, the replication timing of these regions, TERRA/TelRNA expression and the abnormal telomeric phenotype in ICF syndrome.

RESULTS

Subtelomeric regions in ICF cells are hypomethylated

In order to investigate whether subtelomeric regions are hypomethylated in ICF syndrome similarly to other repetitive sequences, we analyzed the methylation status of human subtelomeric regions in LCLs from three ICF patients (pCor, pG and pY). We examined subtelomeric-specific repeats that are known to be hypomethylated in germ cells and highly methylated in somatic cells (9–11). Two such repeats were studied, Hutel (10) and NBL1 (47), and the clear methylation difference of these sequences between sperm and cord-blood DNAs can be observed in Figure 1A and B. Comparison of the hybridization patterns in ICF cells with that of sperm DNA indicates that the methylation status of these repeats in the three ICF LCLs is similar to that in sperm. For patient pCor, the parental LCL controls were available ([Cor (father) and mCor (mother)], and they showed the expected high methylation levels, which are similar to those of cord-blood DNA (Fig. 1). In view of previous finding that described general genomic demethylation and specific hypomethylation of satellite 2 and 3 sequences in LCLs (48), we studied the subtelomeric methylation status in several additional control LCLs that were cultured continuously for several months. In an additional three non-ICF LCLs, these regions were heavily methylated, similarly to the cord-blood DNA (Supplementary Material, S1), suggesting that the subtelomeric hypomethylation is specific for ICF-LCLs. To further confirm the results derived from the ICF LCLs, we analyzed the subtelomeric methylation status in ICF pCor primary fibroblasts. These cells were also hypomethylated in the NBL-1 and Hutel subtelomeric repeats (Fig. 1). Thus the subtelomeric repeats behave in a similar fashion to other repetitive sequences in ICF cells, displaying an early embryonic or germ-cell-like pattern of methylation (13,15,16,38,49,50).

An additional approach to evaluate methylation of subtelomeric regions is terminal restriction fragment (TRF) analysis of genomic DNA digested with the MspI (methylation insensitive) versus the HpaII (methylation sensitive) isochizomers. The size difference between the mean telomere lengths produced by MspI in comparison with HpaII (ΔH–M) reflects the degree of methylation at the subtelomeric regions. We performed such an analysis, quantitated and compared the mean TRF size obtained with each of the restriction enzymes. In the fCor and mCor control LCLs, the ΔH–M is 3.1 and 2.3 kb, respectively (Fig. 2A and B). This difference is shrunk in the three ICF LCL samples indicating hypomethylation of the subtelomeres in the ICF LCLs. In pCor, the offspring of the normal controls, ΔH–M is 1.2 kb, and in pG, pY and the pCor primary fibroblasts almost no size difference was observed in the TRF mean length values using HpaII and MspI digestions (0.4, 0.2 and 0.1 kb, respectively) (Fig. 2A and B) indicating that in these cells the extreme distal
regions of the subtelomeres are almost devoid of cytosine methylation in as much as methylation at MspI/HpaII restriction sites is indicative of total CpG methylation.

Telomere length in ICF syndrome is abnormally reduced both in telomerase-positive and -negative cells

In addition to the methylation status, the TRF analysis with MspI clearly indicated that the mean telomere length of the ICF patients was very short. In the case of pCor, it is possible to compare the telomere length with parental telomere lengths (Fig. 2A and B), and although the pCor LCL was established from the patient at 1 year of age, compared with ages 30 and 26 of her father and mother, respectively (http://www.coriell.org), the telomere length in these cells was shorter than the parental controls. To confirm the mean telomere lengths by an additional methylation-insensitive restriction enzyme, all LCL DNA samples were digested with HinfI, and TRF analysis was performed (Fig. 2C). Mean telomere lengths obtained by HinfI digestion were 8.7, 7.1 and 5.2 kb for the father (fCor), mother (mCor) and ICF (pCor) LCL DNA, respectively, thus confirming the result obtained with MspI-digested DNA. HinfI digestion of LCL DNAs from pG and pY indicated, similar to pCor, a short mean telomere length (Fig. 2C and D).

Study of telomere length in LCLs must take into account the status of telomerase expression in these cells which can influence telomere length independently and with no association with mutations in DNMT3B. Since not all LCLs express telomerase (51–55) and the ICF LCLs may have been passaged more than the control LCLs, it was essential to determine whether all the LCLs exhibit telomerase activity. To this end, we performed a telomeric repeat amplification protocol (TRAP assay) on the ICF and control LCLs which indicated that telomerase activity was present in all the cell lines examined (Fig. 2E). This indicates that although telomerase is active in all the studied LCLs, it does not maintain telomere length with a similar efficiency in the ICF LCLs as in normal control LCLs.

The telomere length in the ICF pCor fibroblast cells was also short (Fig. 2A and B), however, in human primary fibroblasts there is no telomerase activity that maintains telomere length (56 and see Supplementary Material, S2), and shortening can be due to accumulating cell divisions in culture. Since no fibroblasts were available from pCor’s parents, we compared the telomere length of pCor fibroblasts with the telomere lengths of three primary fibroblast lines established from newborn foreskins. TRF analysis of HinfI-digested DNA of pCor at population doubling (PD) 19 clearly indicated that the telomere length in these cells is shorter in comparison with all three controls even though their DNA was extracted at more advanced PDs (Fig. 2F and G). This finding suggests that accelerated telomere loss occurs in ICF fibroblasts.
Figure 2. Mean telomere length in ICF syndrome is abnormally short both in telomerase-positive and -negative cells. (A) TRF analysis was performed on DNA extracted from the three ICF LCLs (pCor, pG and pY), from ICF primary fibroblasts (pCor fib.) and from control LCLs derived from the father (fCor) and mother (mCor) of pCor. DNA was digested with HpaII (H) or MspI (M). Southern blot analysis was performed with a (TTAGGG)₃ probe. Size markers in kb appear on the left. (B) Mean telomere length in kb as determined by several repeats of the Southern blots described in 2A. Blots were analyzed by MATELO and the mean telomere length in kb is displayed below the graph. Black bars display HpaII-determined mean telomere length and white bars – the MspI-determined mean telomere length. n = number of repeated Southern analyses. (C) TRF analysis of the LCL samples of ICF patients and pCor parental controls (fCor and mCor) with HinfI-restriction enzyme. Size markers in kb appear on the left. (D) Mean telomere length in kb as determined by several repeats of the HinfI-TRF analysis followed by MATELO analysis. (E) TRAP assay analysis of the three ICF LCLs, parental controls (fCor and mCor) and positive and negative controls provided by kit. H.I., heat inactivation. Upper panel shows TRAP assay results for pCor, parental and positive control. Lower panel shows TRAP assay results for the three ICF LCLs and positive and negative controls. (F) TRF analysis of DNA digested with HinfI from ICF pCor fibroblasts and several control primary fibroblast lines. (G) Mean telomere length in kb as determined by several repeated experiments (n = number of repeats). The PD at which the DNA was extracted from the cells appears below.
Telomeres in ICF-lymphoblastoid cells exhibit elevated rates of SFEs

TRF analysis indicated that on the level of a whole cell population, the telomeres of ICF cells are abnormally short. This finding prompted us to analyze the occurrence of SFEs at individual telomeres following hybridization of a telomeric probe to metaphase spreads. A previous study on ICF LCLs detected elevated rates of SFEs, however, those cells were assumed to lack telomerase activity (42). Metaphases from the ICF patients’ LCLs and the control LCLs, all of which we had shown to have telomerase activity, were hybridized with a telomere-peptide nucleic acid (PNA) probe. 1600 chromosome ends were scored for hybridization to the telomere probe. We scored for two types of SFEs – those where only one of the sister-chromatids lacked a signal, previously described as STL (sister telomere loss) (57) and those lacking signals from both sister-chromatids which we termed SFE (Fig. 3A). Indeed, the ICF LCLs exhibited a significantly elevated frequency of both STL and SFEs in comparison with the control LCLs (Fig. 3B), however the degree of STLs and SFEs varied among the three patients’ LCLs.

T-SCE is not elevated in ICF cells

Gonzalo et al. (25) described the phenotype of mES null for Dnmt3a and 3b. These cells exhibited hypomethylation in sub-telomeric regions together with abnormally long telomeres. This observation of telomere lengthening in the Dnmt3a/b−/− mES cells was attributed to elevated sister-chromatid exchange at telomeric regions (T-SCE). The occurrence of T-SCE can be assessed using the chromosome orientation (CO)-FISH assay (58–60). CO-FISH hybridization normally displays only one hybridization signal per two sister-chromatids at a chromosome end and will show a single signal. When T-SCE occurs after replication, splitting of the signal between both chromatids occurs and a DS will be detected. The intensities of the two signals may not be symmetric, depending on the site of exchange within the telomere. (B) A partial metaphase of an ICF LCL cell (pCor) following CO-FISH with the PNA-telomere probe. The chromosome at the right upper corner displays T-SCE. (C) Quantitation of T-SCE in the control (3125, fCor, mCor LCLs and FSE+ hTERT fibroblasts) and ICF (pCor, pY, pG LCLs, pCor fibroblasts (pCor fib.), and pCor fibroblasts + hTERT (pCor fib.+ hTERT)) cells. The percentage of chromosome ends displaying DS out of the total screened telomere ends (%T-SCE) is indicated together with the number of these chromosome ends per total chromosome ends screened (n). 20–26 Metaphases were analyzed for each cell line. No significant differences were found between the rate of T-SCE in the ICF cells compared with the control cells (P = 0.9, χ² test with Yate’s correction).
telomeric regions. Applying this approach, we compared T-SCE in the ICF and control LCLs. No significant difference was found between the control and ICF cells (Fig. 4C), and the levels of T-SCE were low and comparable with results reported in other studies (60).

Following T-SCE analysis of the ICF LCLs, we continued and analyzed the pCor fibroblasts. Similar to the ICF LCLs, these cells exhibited low levels of T-SCE, comparable with the levels in control cells. Since the telomere length in ICF cells is very short, occurrence of T-SCE may be underestimated due to very weak hybridization signals. Therefore we elongated the telomeres in pCor fibroblast cells by strong ectopic expression of human telomerase (hTERT). The telomeres in the pCor fib. + hTERT cells were elongated to lengths exceeding 12 kb (Fig. 7A), and subtelomeric regions remained unmethylated, as expected (Supplementary Material, S3). Hybridization of telomeric probe to these cells produced robust signals and CO-FISH did not reveal elevated degrees of T-SCE (Fig. 4C). These findings suggest that the phenotype of the human ICF-syndrome cells differs fundamentally from that of the Dnmt3a/b−/− mES cells and that the hypomethylation of the human subtelomeres in the ICF syndrome is not associated with elevated degrees of T-SCE.

Replication timing of telomeres is advanced in ICF cells

Advanced replication timing of hypomethylated repetitive sequences and foci from the inactive X-chromosome have been observed in ICF cells (35,38, and reviewed in 39). Telomeres are most probably replicated from origins located in the subtelomeric regions, and hypomethylation of these regions in ICF may influence their replication timing. The replicative detargeting FISH (ReDFISH) assay (61) is based on the CO-FISH method and serves to accurately determine the replication timing of telomeres. We modified the ReDFISH assay and performed it on an unsynchronized cell population. We first determined the lengths of G2- and S-phase in the studied cells as described (61,62), and then incubated the asynchronous cells with BrdU for periods that extend longer than G2, with progressive hourly increments (schematic representation in Fig. 5A). Metaphase spreads were prepared from each incubation period, hybridized to the telomere-PNA probe and at least 800 chromosome ends were analyzed from each time point for the presence of single or double hybridization signals. Double signals (DS) indicate that the telomere replicated prior to the addition of BrdU to the culture, and single signals indicate that the telomere replicated after BrdU was added. The earlier the telomeres replicated in S-phase the lower the single-signal percentage would be at shorter BrdU-incubation periods.

Previous findings in ICF LCLs have to be taken into account when performing this assay; a relatively high percentage of chromosome ends is missing a signal from one chromatid due to telomere loss (the STLs) (Fig. 3B). When performing the replication-timing assay, these STLs will be undifferentiated from the telomere ends displaying one signal due to incorporated BrdU and will be erroneously counted as replicated telomeres. Therefore, we performed the replication-timing assay only on the cells of pCor that demonstrated relatively lower levels of STLs (Fig. 3B). We first tested whether similar STL values were obtained when performing CO-FISH on chromosomes that were not exposed to BrdU that normally are expected to exhibit a DS, one signal for each sister-chromatid. This hybridization yielded values of 7.3% in the ICF LCL and 1.5% in the paternal LCL of chromosome ends displaying a STL, which are not a consequence of telomere replication. Consequently, these values were considered as the background levels of the single ‘replicated’ pattern, and were subtracted from values obtained in the replication-timing assay. Following this adjustment, the maximal values of replicated ends could not exceed 92.7% in ICF and 98.5% in the paternal control cells. Very low levels of T-SCE (Fig. 4C) produced signals on both sister-chromatids such that the final values of ‘replicated telomeres’ were further reduced.

The length of G2 was determined as 4–4.5 h and S-phase as ~10 h in both ICF and paternal LCLs. Incubation periods with BrdU ranged from 4 to 12 h continuously with two additional time points of 15 and 18 h of BrdU incubation. As shown in Figure 5B and Supplementary Material, S4, the single-signal pattern, indicative of replication, occurred at significantly lower levels in the ICF cells at shorter periods of BrdU labeling.
This observation indicates that the telomeres replicated earlier in S-phase in ICF, compared with the control LCL. For example, ~21% of single signals were scored at 8 h of BrdU incubation in the ICF cells, in comparison with 55% in control cells. Only at 11 h of incubation ~55% of telomere ends displayed single signals in the ICF cells. Thus, ~45% of the telomeres had replicated in the ICF cells 3 h earlier in S-phase compared with control cells. These values also indicate that in a normal LCL, >50% of the telomeres replicate during the last 3–4 h of S-phase. Hence, while the replication of telomeres is distributed throughout S-phase, with some telomeres replicating also early in S-phase, the majority of telomeres replicate during the last half to third of S-phase. In comparison, >50% of telomeres in the ICF LCL replicate during the first third to half of S-phase. Of note, the differences in telomere replication timing cannot be attributed to a prolonged S-phase in the ICF LCL, since the length of S and G2 phases were found to be comparable.

Transcripts originating from telomeric regions are present at significantly higher levels in ICF cells in comparison with control cells

Recent studies have shown that mammalian telomeric regions are transcribed into TERRA (7), described also as TelRNA (8). These transcripts initiate in human cells from within several subtelomeric regions, and are composed of subtelomeric sequences and UUAGGG repeats (7,8). As hypomethylation and advanced replication are associated with transcriptional activation (reviewed in 63) we asked whether the hypomethylation and advanced replication timing of the subtelomeric regions in ICF might be accompanied by enhanced TERRA/TelRNA transcription. To this end, northern blot analysis with the C-rich telomere probe was carried out on total RNA isolated from pCor and paternal fCor LCLs. As shown in Figure 6A the levels of TERRA/TelRNA are significantly higher in the patient sample when compared with the...
paternal control, and as expected, no hybridization was obtained when the G-rich telomere probe was used. This northern analysis was repeated with RNA from two additional ICF LCLs, pCor’s maternal control, mCor, and several additional control RNAs (Fig. 6B), and the telomere-transcript levels were calibrated relative to β-actin hybridization signals. The telomere transcripts were present at 3–8-fold higher levels in the pCor LCL in comparison with the parental controls and compared with three additional control RNA samples (Fig. 6C).

In order to examine whether these transcripts are elevated in other cell types in ICF syndrome, northern analysis was also performed on total RNA from pCor primary fibroblast cells and two control primary fibroblast cell lines. Similarly to the results obtained with the LCLs, the expression of transcripts in the ICF fibroblasts was clearly higher than the expression of these transcripts in the control fibroblasts (Fig. 6D).

To confirm that the northern hybridization signals, present as a smear, indeed represent transcripts emanating from chromosome ends and not from internal telomeric repeats, we isolated RNA from the pCor fibroblasts at different stages following the introduction of hTERT. Ectopic hTERT was incorporated in many copies in these cells resulting in high levels of hTERT expression as demonstrated by TRAP assay (Supplementary Material, S2). As shown by TRF analysis in Figure 7A, the telomeres elongated gradually after the introduction of the hTERT. Concomitantly, the length of the TERRA/TelRNA hybridization smear increased in size as demonstrated by northern analysis (Fig. 7B), validating the source of this signal from telomeric repeats at chromosome ends. The hybridization intensity of the TERRA/TelRNA was not affected significantly by the change in telomeric length (as determined by phosphorimage analysis) or by the levels of telomerase expression in these cells. These findings suggest that DNA hypomethylation at subtelomeric regions in ICF cells is indeed accompanied by prominent transcription emanating from these regions.

**DISCUSSION**

The investigation of ICF syndrome has elucidated many basic epigenetic events that occur in mammalian cells during development and adult life. The mutated gene responsible for approximately half of the reported cases of this syndrome is DNMT3B (26–31). Numerous studies, carried out in murine models, have demonstrated the importance of Dnmt3b in de novo methylation events during early mammalian development, and later in life in maintaining the DNA methylation patterns (21, 24, 28, 64). The fact that 70% of the mammalian genome undergoes methylation (65) may explain the pleiotropic range of phenotypes associated with this disease. One of the major phenotypes observed in ICF syndrome is the cytogenetic abnormality at the centromeres of chromosomes 1, 9 and 16, which lead to marked chromosomal instability (12, 16, 31, 42). Here we show that additional chromosomal sites affected in ICF syndrome are the subtelomeric regions, suggesting that hypomethylation of these regions may play an important role in the chromosomal instability observed in this disease.

Repetitive sequences are important targets of DNMT3B (66, 67). In addition, DNMT3B is characterized by high processivity and is well suited to methylate CG-rich regions (67). Human subtelomeric regions are CG-rich and contain several repetitive sequences (9–11). We therefore examined the methylation status of the subtelomeric regions in cells derived from three ICF patients. The mutations in the DNMT3B gene in pCor and pG have been described previously (27, 28, 31), and pY carries a homozygous mutation in the catalytic domain of DNMT3B (unpublished data). Mouse models carrying mutations identical to those of pCor [mouse mutations A609T and STP813 corresponding to human mutation A603T and the 9 bp insertion at codon 744 (28)], demonstrate partial loss of function and phenotypes resembling those of ICF syndrome (24). DNA methylation analysis confirmed that indeed subtelomeric regions are hypomethylated in LCLs from all three ICF patients, and in primary fibroblasts of pCor (Figs 1 and 2) to degrees comparable with those in sperm (Fig. 1A and B). While the results of the TRF analysis are general for all subtelomeric regions, the analyses of the repetitive sequences Hutel and NBL1 are specific for a subset of CpG sites at these repeats. The highly CG-rich Hutel repeat (10) described by several groups [pTH2 (11), TelSau2.0 (68), HSY (69)] is located, according to in situ hybridization, on at least 50% of the subtelomeric regions (10), while the NBL1 sequence is positioned adjacent to the p-arm telomeres of the acrocentric chromosomes and in addition near the centromeres of chromosomes 3 and 4 (47). The subtelomeric
hypomethylation in ICF cells is in concordance with the hypomethylation observed in several subtelomeric regions in the Dnmt3a/b<sup>−/−</sup> mES cells (25), however, the ICF cells clearly demonstrate that mutations in Dnmt3B alone suffice to impair subtelomeric methylation. Clearly, human subtelomeric regions, at least the distal regions adjacent to the telomeric repeats, constitute an additional target of Dnmt3B, and the residing activity of Dnmt3B in ICF syndrome (24) is not sufficient to maintain normal methylation of these regions.

In the Dnmt3a/b<sup>−/−</sup> mES cells, the telomeres were found to be abnormally long, and this phenotype was attributed to increased T-SCE due to the hypomethylated state of the subtelomeric regions (25). In contrast to the findings in Dnmt3a/b<sup>−/−</sup> mES cells, telomere length was found to be abnormally short both in the three ICF LCLs and the primary fibroblasts of pCor (Fig. 2). In concordance with the human ICF cells, Dnmt3b<sup>−/−</sup> MEFs (mouse embryonic fibroblasts) show abnormalities, such as end-to-end fusions and aneuploidy (64). In primary fibroblasts, where telomerase is not expressed at levels that maintain telomere length (56), the excessive shortening of the pCor fibroblasts in comparison with normal fibroblasts at even higher PDs (Fig. 2F and G) indicates that accelerated telomere shortening occurs in ICF fibroblasts. However, when expressed ectopically at high levels, telomerase is capable of elongating telomeres in ICF fibroblasts (Fig. 7A), thus overcoming the yet uncharacterized abnormality that results in telomere shortening.

In LCLs it is more difficult to determine the cause of telomere shortening. If telomerase is not expressed in an LCL, the short telomere length may be solely the result of the continuous passing in culture (51,53,54). In the current study the ICF and control LCLs, all showed similar easily detectable levels of telomerase activity by TRAP assay (Fig. 2E). Although this does not offer conclusive proof for the functional elongating activity in the living cells (70,71), it does suggest that the abnormally short telomere length in the ICF cell line is not merely the consequence of continuous passing in culture. The combined analysis of both the ICF LCLs and fibroblasts suggests that the short telomere phenotype can be dissociated from the status of telomerase expression and that the telomere-length phenotype resulting from loss of function of the Dnmt3B gene product differs between mES cells on one hand and human ICF LCLs and fibroblasts on the other hand.

Additional information on the process of telomere loss can be inferred from the FISH studies. The striking high frequency of STLs in pY and pG indicates that most of the observed telomere loss occurs in only one of the two sister-chromatids, information that could not be inferred from TRF analysis alone. Another finding that stemmed from the hybridization analysis is reflected in the standard deviation values is that the degrees of STL and SFE varied among different cells in a culture indicating that the telomere loss likely occurs via a gradual rather than an abrupt process.

T-SCE has been shown to be markedly affected by epigenetic modifications at telomeric regions in both mES and MEF cells (25,72). However, while Dnmt3a/b<sup>−/−</sup> mES cells demonstrated elevated rates of T-SCE, the levels of T-SCE in the ICF LCLs were low and comparable with the levels observed in the control LCLs (Fig. 4). ICF fibroblasts demonstrated low levels of T-SCE as well, and extensive elongation of telomeres in these cells by ectopic expression of hTERT, did not influence the levels of T-SCE (Fig. 4). Thus, in human cells with hypomethylated subtelomeres adjacent to abnormally long telomeres, no elevation in T-SCE is observed. Interestingly, MEFs derived from mice with a mutation in the RNA component of telomerase (Terc<sup>−/−</sup>) are characterized by abnormally short telomeres and hypomethylation at subtelomeres. These cells demonstrate elevated T-SCE as well, and this feature has also been attributed to the hypomethylation of the subtelomeric regions (72). It is unclear why the hypomethylated subtelomeres in the ICF syndrome patients do not elevate the degree of T-SCE. The reason may lie in dissimilarities in subtelomeric and telomeric features between mice and human cells with particular reference to the substantial difference in telomere length between laboratory mice in comparison with human telomeres. Notably, the abnormally short length of the telomeres in the G5 generation of the Terc<sup>−/−</sup> mice that exhibited elevated T-SCE was still >20 kb (72) while the length of the ICF telomeres was in the range of 4–5 kb.

The phenotypic discrepancies in telomere length and function between the Dnmt3a/b<sup>−/−</sup> mES cells and other cells raise the possibility that the phenotype associated with Dnmt3B loss-of-function differs in cells according to the developmental stage from which they originate. mES cells are derived from embryos at about E4.0 and resemble most closely epiblasts (73,74). At this embryonic stage, DNA methylation in the inner cell mass is undetectable (75). More specifically, minor satellite sequences are unmethylated at this preimplantation stage (76) and are methylated de novo by Dnmt3b shortly after embryonic implantation (77). In human sperm and somatic cells (10,11) and in mES (25,78), subtelomeric repeats behaved similarly to minor satellite DNA with respect to their methylation status. Most probably, the subtelomeric regions are also unmethylated in the inner cell mass in vivo, and are de novo methylated by Dnmt3b only after implantation. If this is indeed the case, in vitro culturing of ES (embryonic stem) cells induces methylation of repetitive sequences. Similarly, many human undifferentiated female ES cells undergo X-chromosome inactivation in culture (79,80). We have found that human ES cells, similar to mES cells, are also methylated at subtelomeric regions, both in the undifferentiated pluripotent state and following spontaneous differentiation in culture (data not shown). Following this reasoning, ES cells may be more tolerant to the various methylation states of repetitive sequences than differentiated cells. Indeed, Dnmt3a/b<sup>−/−</sup> mES cells are viable, while mouse embryos null for Dnmt3b die at day 13.5 of development (28), and Dnmt3b-deficient MEF cells either enter senescence prematurely or are immortalized spontaneously with accompanying karyotypic instability (64). Another possibility is that other epigenetic characteristics of ES cells are sufficient to induce the elevated T-SCE in the Dnmt3a/b<sup>−/−</sup> mES cells, leading to the abnormally long telomeres. T-SCE has been shown to be a dominant mechanism for elongating telomeres during early cleavage embryos (81) and although at the blastocyst stage, at which ES cells are derived from, T-SCE is reduced and telomerase is activated, higher levels of T-SCE in comparison with somatic cells may still persist. A clear difference in the binding pattern of
Dnmt3b in mES in comparison with MEFs has also been observed (82), suggesting that the roles that this enzyme plays at different developmental stages may be relevant also to the different telomeric phenotypes observed when DNMT3B is absent. Hence, while it is clear that subtelomeric methylation plays an important role in controlling telomere length and function (6,25), the effect of hypomethylation may differ in cells at different development stages or from different organisms.

Hypomethylation has been strongly correlated with earlier replication timing in S-phase (63,83). In ICF syndrome, advanced replication timing of hypomethylated regions has been demonstrated in the case of the satellite 2 repeats (38) and several sequences on the inactive X-chromosome (35). In the current study, analysis of telomere replication timing in a normal LCL, revealed that the majority of the telomeres replicated during the last half to third of S-phase, as previously reported (84). This is expected, as heterochromatic regions are known to replicate late in S-phase (85). In contrast, in the pCor ICF cells, the replication timing of telomeric regions as a group is advanced by ~3 h (Fig. 5B) implicating the state of methylation in the control of replication timing of this region. Previously it has been suggested, at least in the case of imprinted regions, that the first epigenetic marking during development is in the form of asynchronous replication, followed only later by differential DNA methylation patterns (86). At this point it is unknown whether early in ICF syndrome development, these regions replicate late in S-phase and consequently their hypomethylated state induces earlier replication timing. In *Saccharomyces cerevisiae* (*S. cerevisiae*), abnormally short telomeres are earlier replicating due to activation of more proximal earlier-firing origins of replication (87). Whether this is the case in ICF syndrome or whether the conventional origins of replication shift their firing due to hypomethylation, is an important question for further study.

What potential consequences could advanced replication timing of the subtelomeric and telomeric regions have? Both hypomethylation and early replication are strongly correlated with transcription (63). In the case of genes on the ICF-inactive X-chromosome, advanced replication timing was the critical factor that determined escape from gene silencing (35). Transcription of normally repressed DNA sequences has also been observed in ICF LCLs in the case of repetitive sequences. Satellite 2 repeats are expressed in some ICF LCLs (88) and as noted earlier have been also observed to advance their replication timing in S-phase (38). Another example of a transcribed repetitive non-satellite sequence in ICF LCLs is the NBL2 repeat (13). Transcription of subtelomeric repeats has been demonstrated in a previous study (69) and more recently it was demonstrated that mammalian telomeres are also transcriptionally active. TERRA/TelRNA initiates from within several subtelomeres and contains G-rich RNA transcripts transcribed on the leading strand template (7,8). Here we show that TERRA/TelRNA expression is dramatically enhanced in the ICF LCLs and fibroblast cells, while all control RNAs exhibited very low levels of these transcripts (Fig. 6). The high levels of TERRA/TelRNA in ICF cells are in contrast to Dnmt3a/b−/− mES, in which levels of TelRNA were slightly reduced (8). This again may reflect a fundamental biological difference between ES cells and differentiated somatic cells. However, preliminary results in human ES cells showed elevated levels of TERRA/TelRNA (data not shown). Thus, this discrepancy may reflect an organism-based difference. Reduced levels of TelRNA was also found to be correlated with reduced telomere length in mice primary and immortalized MEFs (8), while in ICF cells no such correlation was observed. Another discrepancy relates to the change in TelRNA size in concordance to telomere length. Elongation of telomeres in ICF fibroblasts by hTERT resulted in elongated TERRA/TelRNA transcripts (Fig. 7), while TelRNA was found in another study to be maximally 6 kb even in murine cells with very long telomeres (8).

Depletion of components of the nonsense-mediated RNA decay machinery, which plays an essential role in TERRA regulation, leads to telomeric aberrations in several forms, including telomere shortening and SFEs involving either one or both chromatids (7). These telomeric phenotypes are similar to the observed telomere SFEs and STL in the ICF telomeres. Telomere shortening was also observed in *S. cerevisiae* when high rates of telomere transcription were artificially induced (89). Thus, it is tempting to speculate that TERRA/TelRNA abnormalities in ICF syndrome are the link between the hypomethylation of subtelomeric regions and telomere shortening in this disease. As TERRA/TelRNA was found to localize to the telomeric regions (7,8), the strikingly high levels in ICF cells may disrupt the normal process of DNA replication of telomeres by binding to the leading strand and obstructing the movement of the replication fork on this strand, subsequently leading to telomere loss. This model predicts that accelerated telomere loss will occur both in telomere positive and negative cells. However in cells, which express telomerase, such as the LCLs, telomerase will maintain telomeres at a critical short length sufficient to enable the cells to continue proliferating. In fibroblasts, the accelerated telomere shortening should lead to replicative senescence at an abnormally low PD.

ICF syndrome is a very rare genetic syndrome; ~50 patients have been reported so far in the literature (32). Notwithstanding the rarity of this unfortunate disorder, understanding its molecular basis even in isolated cases has shed light on important epigenetic events occurring during development and later in adult life. The telomeric phenotype in this disease is emerging as an important component of the pleiotropic effects of DNMT3B-related-hypomethylation abnormalities on the mammalian genome. Further studies related to the intriguing overexpression of TERRA/TelRNA in this disease, and perhaps of additional repetitive-sequence regions, will allow additional insights into the role of the methylation and expression of repetitive sequences in general and subtelomeric regions in particular in normal development and in disease.

**MATERIALS AND METHODS**

**Patients and controls**

Three ICF patients were studied. A LCL (GM08714) and primary fibroblasts (GM08747) from a female patient, designated here pCor, were obtained from the Coriell Institute for Medical Research, Camden, NJ, USA (http://www.coriell.org). This patient is a compound heterozygote, her mother is...
a heterozygote carrier and her father transferred a de novo germ-line mutation (28, 41). LCLs from the father (GM08729), designated here fCor, and mother (GM08728), designated here mCor, were also obtained from the Coriell Institute for Medical Research.

An additional LCL from a male ICF-syndrome patient G (pG) was described previously (31). This patient is a compound heterozygote, as well. A third LCL from a male ICF-syndrome patient Y (pY) carries a homozygous mutation in the catalytic domain of DNMT3B (unpublished data). Additional control LCLs included 3133, 3125, NT (90) and GM07078 (Nijmegen breakage syndrome, NBS1) obtained from Coriell Institute for Medical Research. Primary fibroblast controls included WI38, FS1, FS3, FSE and FSE + hTERT, which was immortalized by ectopic expression of human telomerase.

**Cell culture**

LCLs were grown in RPMI supplemented with 15% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The ICF LCLs were grown in similar media supplemented with 20% FCS. The primary fibroblast cells originating from pCor were obtained at PD 14.8 (information from Coriell Institute for Medical Research) and were grown in MEM media supplemented with 20% FCS, glutamine and antibiotics, as described earlier. Control primary fibroblast cultures were grown in DMEM media supplemented with 10% FCS, glutamine and antibiotics.

**Retroviral infections**

FSE and pCor fibroblasts were serially infected with a pBABE–eGFP construct containing the human telomerase gene, as described previously (91). These cells were designated FSE + hTERT and pCor fib. + hTERT.

**DNA-methylation and TRF analysis**

Genomic DNA was extracted according to standard procedures. Sperm DNA was extracted as described (84). Purified DNA was subjected to restriction enzyme digestion and gel electrophoresis. DNA was transferred to a MagnaGraph nylon transfer membrane (Water and Process Technologies) and hybridized with the appropriate probe and conditions as described later.

Subtelomere-methylation analysis was conducted with two subtelomere repeat-probes: analysis with the Hutel probe (10) was carried out using DNA digested with MboI and Sau3A1, and separated on a 1% agarose gel. Analysis with the NBL1 probe (47) was carried out using DNA digested with MspI and HpaII and separated on a 2% agarose gel. The NBL1 probe was generated by PCR amplification of human genomic DNA, using primers based on the NBL1 sequence (GenBank accession no. U53226) and subsequently cloning the PCR product. The primers used for this cloning were – NBL1-FOR: 5'-CCCGGAAGATTCTGATAC-3' and NBL1-REV: 5'-GATTTGGTATCTGGAGCC-3'. The annealing temperature was 56°C. Hybridization was carried out as described (84).

Telomerase activity was measured in 600 ng protein extract using the TRAPEZE® ELISA Telomerase Detection Kit S7750 or TRAPEZE® Telomerase Detection Kit S7700 (Chemicon International, Canada). TRAP assay products were visualized on a 12.5% polyacrylamide gel following staining with ethidium-bromide.

**Telomere SFE analysis**

Metaphase chromosomes for SFE analysis were obtained by standard procedures after treating the cultures with 0.04 µg/ml colcemid for 1 h. Hybridization was performed with a PNA-((CCCTAA)3 oligo conjugated to Cy3 as described (93–95).

Eighteen or more metaphases were analyzed, yielding cumulatively a total of >1600 scored chromosome ends. The signals were scored for the presence of two (DS), 1 (STL) or absent (SFE) telomere-hybridization signals.

**Chromosome orientation-fluorescence in situ hybridization**

Cells were grown in the presence of 2.5 × 10^-4 M BrdU for various incubation periods. Colcemid was added at a concentration of 0.04 µg/ml during the last hour of the BrdU-incubation period. Metaphase spreads were prepared by standard procedures. CO-FISH was essentially performed as described (96) with a few modifications: slides were treated with 1 mg/ml of Hoechst 33258 for 15 min followed by exposure to 365 nm UV light for 30 min using a UV Stratalinker 1800 transilluminator (STRATAGENE). The probe used for hybridization in the CO-FISH experiments was the PNA-telomere probe described earlier.

**Telomere sister-chromatid exchange**

T-SCEx exchange was determined by performing CO-FISH on chromosomes grown in the presence of BrdU for 18–24 h. This period covers the entire S + G2 phases in LCL, therefore each chromosome end is expected to display a telomere signal from only one of the two sister-chromatids (59). In cases of T-SCEx both sister-chromatids will hybridize to the telomeric probe, i.e. a ‘split’ telomere CO-FISH signal (59, 60). The hybridization was performed with the PNA-telomere probe. Over 1700 telomere ends were analyzed from a minimum of 20 metaphases, and the percentage of telomere ends displaying DS was determined from the total telomere ends displaying either one or two signals.
Telomere replication-timing assay

Telomere replication timing was determined by a modification of the RedFISH assay (59,61). The analyzed cells were labeled with BrdU for different time intervals, and for the last hour of labeling were incubated with 0.04 μg/ml colcemid. Metaphase spreads were prepared and CO-FISH was performed with PNA-telomeric probe as described earlier. For each time point 800–2150 chromosome ends displaying either a single signal or DS were scored and the percentages of single and double telomere signals were determined. For determination of the background of single hybridization signals, cells that were grown in the absence of BrdU were hybridized to the telomere probe with the CO-FISH conditions. When no BrdU labeling is performed, each chromosome end is expected to display DS, unless telomere length is critically short in a subset of chromatids. This single-signal background was subtracted from all time points in the replication-timing assay.

The length of G2- and S-phase in the analyzed LCLs was determined as described (61,62). The BrdU labeling of mitotic chromosomes was determined by immunostaining with an anti-BrdU antibody (MS-1058-P, NeoMarkers, Fremont, CA, USA).

Fluorescence microscopy

Fluorescence hybridization was visualized using a BX50 microscope (Olympus). Images were captured on the Olympus microscope with an Olympus DP70 camera controlled by DP controller software (Olympus). Analysis and pseudocolor rendering were conducted using DP manager software (Olympus). DAPI was pseudo colored in red and CY3 in green.

Northern blot analysis

RNA was extracted with TRI REAGENT (Molecular Research Center, Inc., Cincinnati, OH, USA). Twenty μg of RNA were subjected to electrophoresis on 1.2% agarose gel and transferred to a MagnaGraph nylon transfer membrane. To detect transcripts from telomeric regions, membranes were hybridized either to a 32P-end-labeled (TAACCC)3 oligo or a (TTAGGG)3 oligo using the conditions described earlier for TRF blot analysis. In order to normalize signals arising from telomeric probes, the northern blots were re-hybridized to a human β-actin probe (97). Hybridization signals were quantitated by phosphorimager analysis.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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