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Whole-genome methylation scan in ICF syndrome: hypomethylation of non-satellite DNA repeats *D4Z4* and *NBL2*

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The ICF (immunodeficiency, centromeric instability and facial abnormalities) syndrome is a rare recessive disease characterized by immunodeficiency, extraordinary instability of certain heterochromatin regions and mutations in the gene encoding DNA methyltransferase 3B. In this syndrome, chromosomes 1 and 16 are demethylated in their centromere-adjacent (juxtacentromeric) heterochromatin, the same regions that are highly unstable in mitogen-treated ICF lymphocytes and B cell lines. We investigated the methylation abnormalities in CpG islands of B cell lines from four ICF patients and their unaffected parents. Genomic DNA digested with a CpG methylation-sensitive restriction enzyme was subjected to two-dimensional gel electrophoresis. Most of the restriction fragments were identical in the digests from the patients and controls, indicating that the methylation abnormality in ICF is restricted to a small portion of the genome. However, ICF DNA digests prominently displayed multicopy fragments absent in controls. We cloned and sequenced several of the affected DNA fragments and found that the non-satellite repeats *D4Z4* and *NBL2* were strongly hypomethylated in all four patients, as compared with their unaffected parents. The high degree of methylation of *D4Z4* that we observed in normal cells may be related to the postulated role of this DNA repeat in position effect variegation in facioscapulohumeral muscular dystrophy and might also pertain to abnormal gene expression in ICF. In addition, our finding of consistent hypomethylation and overexpression of *NBL2* repeats in ICF samples suggests derangement of methylation-regulated expression of this sequence in the ICF syndrome.

INTRODUCTION

ICF (immunodeficiency, centromeric instability and facial abnormalities) is a rare autosomal recessive syndrome characterized by the presence of variable immunodeficiency and a unique type of instability of pericentromeric heterochromatin (heterochromatin in the vicinity of the centromere) (1,2). ICF patients have severe recurrent respiratory infections attributable to an immunoglobulin deficiency with or without defective cellular immunity. Facial anomalies and psychomotor retardation are also observed frequently in this syndrome, and various other symptoms such as mental retardation and digestive disorders are often, but not invariantly, seen. The chromosomal abnormalities in this syndrome are an extraordinary collection of stretching, breakage and association of pericentromeric heterochromatin of chromosomes 1 and 16, and, to a lesser extent, chromosome 9. These abnormalities include high frequencies of occurrence of multibranched chromosomes consisting of 3–12 arms of the above three chromosomes joined in the pericentromeric region (2–4).

The unusually decondensed heterochromatin has been localized to the juxtacentromeric (centromere-adjacent) heterochromatin of chromosomes 1, 16 and, less frequently, 9. These are the same regions that have been shown to be abnormally hypomethylated in all studied ICF tissues and cell cultures (5–7). Similar chromosome rearrangements are observed in normal, mitogen-stimulated lymphocytes treated with the demethylating agent, 5-azacytidine, and the same spectrum of abnormalities, including multibranched chromosomes, was seen in a normal pro-B cell line treated with 5-azacytidine or 5-azadeoxycytidine but not with genotoxins that do not induce demethylation of DNA (5,6,8–10).

The above studies indicate that a partial deficiency in DNA methylation is responsible for the diagnostic chromosomal abnormalities in ICF. Indeed, one of three known human DNA methyltransferase genes, *DNMT3B*, which has been mapped to 20q11.2 (11), has been shown recently to be mutated in patients (12–14). This is consistent with the finding that the ICF locus previously had been localized to 20q11–q13 by homozygosity mapping (15). Deficiency of DNMT3B probably accounts for the symptoms associated with this syndrome as well as for abnormal hypomethylation of certain DNA sequences.

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Several specific sequences were reported to be hypomethylated in ICF patients in addition to satellite DNA from the juxtacentromeric heterochromatin of chromosomes 1, 9 and 16. These include Alu sequences, *PGK1* and other X-linked sequences, and, occasionally, *H19* and α-satellite DNA (6,7,10,16–18). However, very little of the genome has been examined with respect to its methylation status in ICF cells, and no transcribed DNA region with consistent ICF-linked hypomethylation like that of juxtacentromeric satellite DNA has been identified (7,10,18).

We have used a whole-genome approach to assess the extent of ICF-specific aberrations in DNA methylation and here identify sequences showing such disease-associated hypomethylation. We compared DNA from ICF patients and their phenotypically normal parents by two-dimensional (2D) gel electrophoresis of genomic digests, a technique which has been used to study genetic variations in the human genome (19,20) and to detect alterations in cancer (21–23). The DNA is digested with a restriction enzyme, end-labeled with [^{32}P]nucleotides and subjected to agarose gel electrophoresis. Subsequently, the DNA fragments in the gels are digested *in situ* with another enzyme and resolved on second dimension gels. More than 2000 DNA fragments can be analyzed simultaneously in patterns from multiple samples, and the use of a CpG methylation-sensitive restriction endonuclease in the first digestion allowed us to study the methylation status of CpG islands (24,25). We have identified, by 2D gel electrophoresis of such digests, DNA sequences with consistently altered methylation status in cells from ICF patients. Several fragments visualized on electrophoresis of these digests differed repeatedly in their occurrence or intensity between the patients and the controls, indicating their ICF-specific hypomethylation. Two of these are repeated sequences that are found in several locations in the human genome: *D4Z4*, whose deletion has been implicated in facio-scapulohumeral muscular dystrophy (FHSD) (26); and *NBL2*, which frequently is hypomethylated in neuroblastomas (21). The latter sequence was found to have increased expression associated with its hypomethylation in cell lines from all three tested ICF patients, as well as in neuroblastoma cell lines.

RESULTS

Comparison of 2D patterns of genomic DNA of B cell lines from ICF patients and their parents

We compared 2D gel patterns of genomic DNA of Epstein–Barr virus (EBV)-transformed B cells from patients with the ICF syndrome and their unaffected parents after digesting the DNA with a CpG methylation-sensitive restriction endonuclease (*Eag*I; CGGCCG) for the first dimension separation, and with a restriction endonuclease indifferent to CpG methylation (*Hin*fI) for resolution in the second dimension. The parents of ICF patients are phenotypically normal and, unlike ICF patients, they have the expected high levels of satellite DNA methylation (6). We analyzed the 2D patterns for consistent differences in DNA fragments of patients and their parents that are attributable to ICF-specific DNA methylation changes and not to restriction fragment length polymorphisms. Also, the use of cell lines from four different ICF families allowed us to distinguish possible random cell culture-associated changes in DNA methylation from ICF-linked methylation anomalies.

Figure 1 shows representative 2D gel patterns of genomic DNA from the analogous B cell lines of ICF patient 4 and her unaffected parents. Digestion with *Eag*I and *Hin*fI yielded >2000 DNA fragments in each pattern. Computer-assisted analysis of 2D patterns was undertaken to detect spots representing DNA fragments that differed in their occurrence or intensity between patients and their parents. The vast majority of spots had the same intensities in ICF patients as in their parents. Most of the spots that exhibited differences between patients and their parents were not common to all patients, as presented in Table 1. They included spots that were present only in the patients or that were of greatly increased intensity in one or more patient's samples relative to those of their parents (Table 1). Conversely, there were also spots that were patient restricted in occurrence or intensity (Table 1). Thirteen spots exhibited differences that were consistent in all patients (Table 1 and Fig. 1). With the exception of fragment 8, all seemed to be derived from multicopy sequences by comparison of the intensity of the spots of unknown origin with the many spots of known unique sequences previously analyzed by the same methods (20). Some of the fragments that exhibited differences in intensity between the patients and their parents may represent related DNA fragments based on the similar migration of these fragments in the first or second dimension (Table 2). Pairs of equally intense fragments with an identical size in the first dimension can result from cleavage at a *Hin*fI site asymmetrically situated between two unmethylated *Eag*I sites in the DNA. This was the case for fragments 2 and 3 and for fragments 6 and 7. Also, a train of fragments with identical (second dimension) *Eag*I–*Hin*fI fragment length can result from variable methylation of *Eag*I sites in repeat units (fragments 1 and 2; 9, 10, 11 and 12) or sequence variation of *Eag*I sites.

Demethylation of the FSHD syndrome-related *D4Z4* repeats in ICF syndrome DNA

All four patients exhibited a fragment with a first dimension size of 2.2 kb and an intensity corresponding to its occurrence in multiple copies in the genome (Fig. 1, spot 1). This fragment was either absent or occurred at a very much reduced intensity in the parents' patterns, suggesting that it was hypomethylated specifically in ICF cells (Table 2). Using *Dpn*II in place of *Hin*fI for the second dimension separation resulted in a pair of high intensity fragments, which had the same first dimension size as fragment 1 and had much greater intensity in the patients than in the parents (data not shown). We sequenced the 0.7 and 0.3 kb fragments from *Eag*I–*Dpn*II double digests of ICF DNA which had the same size in the first dimension (2.2 kb) as fragment 1, which was detected specifically in DNA from ICF patients. Sequencing revealed that the 0.7 and 0.3 kb fragments exactly matched the *D4Z4* repeat at 4q35 (GenBank accession nos AF039145, AF039153 and AF039154). Therefore, for at least 1 kb, the sequence of our two fragments was 100% identical to the 4q35 *D4Z4* repeat. This included the lack of a *Bln*I site which is essentially invariably present in the copies of the *D4Z4* repeat from the highly homologous but not identical tandem repeat on chromosome 10 (27). There is extraordinary homogeneity in the sequence of the 4q35 *D4Z4* repeat (28,29). Such a high degree of homology (100%) as we found between the 0.3 and 0.7 kb fragments and the 4q35 *D4Z4* repeat is unlikely to exist with *D4Z4*-like repeats elsewhere in the genome (30–32), suggesting that the ICF hypomethylated *D4Z4* sequence is derived from the FSHD-associated 4q35 region. The deletion of an integral number of these repeats at 4q35 is thought to be responsible for the FSHD

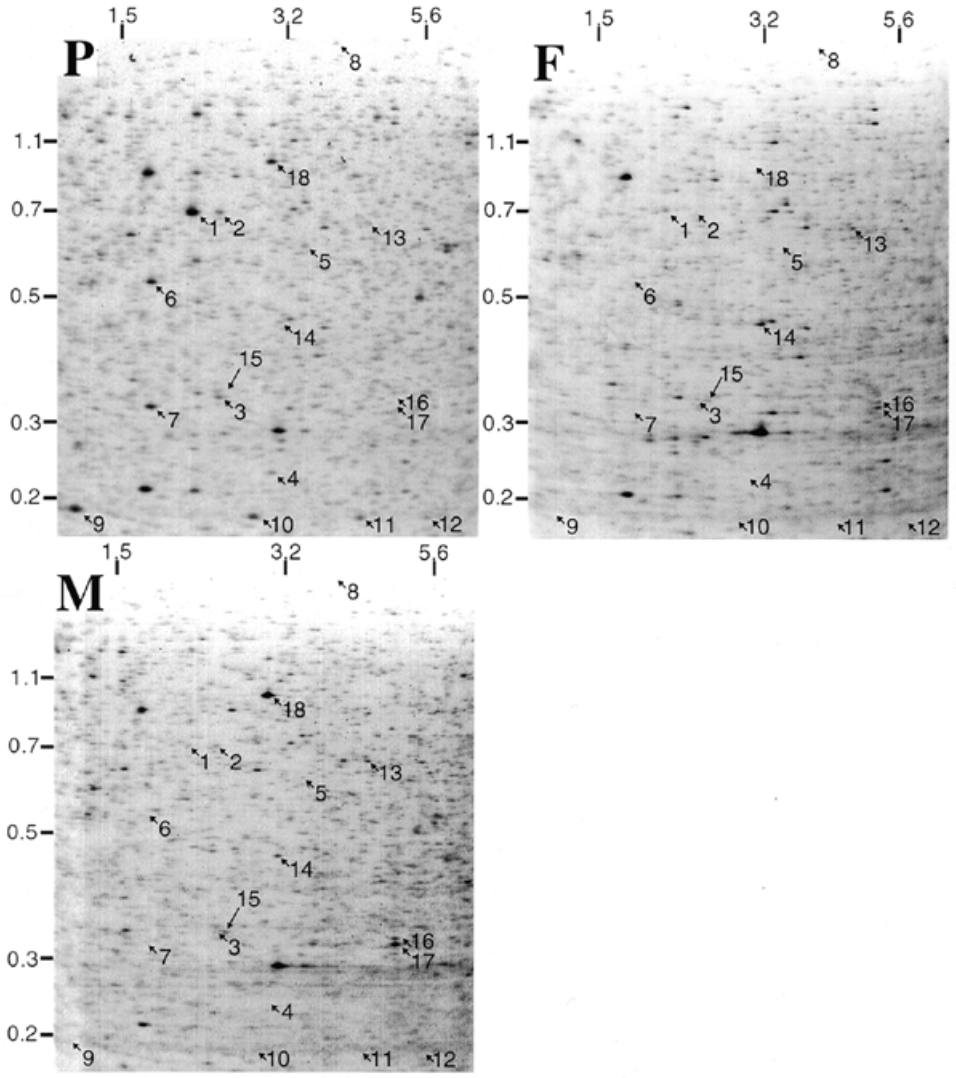

Figure 1. Representative 2D profiles of ICF patients and their unaffected parents. Genomic DNA of B cells from patient 4 and her parents was digested with *Eag*I for first-dimensional separation (left to right of gel) and subsequently digested with *Hin*fI *in situ* prior to second dimension separation (top to bottom of gel). P, patient; F, father; M, mother. The arrows indicate fragments with altered intensities in ICF patients (fragments 1–17). Fragments 1–12 have increased intensities, and fragments 13–17 have decreased intensities in the patients. Fragment 18 represents the GC-rich macrosatellite VNTR in Xq24, which is demethylated specifically on the inactive X chromosome (35).

syndrome (27,30,33). The consensus D4Z4 sequence (30) indicates that *Eag*I and *Hin*fI digestion of *D4Z4* should produce fragment 1 and another fragment (2D size of 86 bp) too small to be visualized on the 2D gels if the *Eag*I sites are fully unmethylated.

To confirm the demethylation of *D4Z4* in ICF, *Eag*I digests of DNA from three ICF families were analyzed by Southern blotting with the cloned *D4Z4* fragments as a hybridization probe. Four hybridizing bands were observed in digests of ICF patient DNAs that had the same first dimension molecular weight as fragments 1–5 in the 2D gels (Fig. 2A). The *Eag*I and *Hin*fI restriction map of *D4Z4* repeats predicts a 2.2 kb band corresponding to fragment 1 and three bands corresponding to fragments 2–5 due to progressive demethylation of *Eag*I sites. It is unlikely that sequence variation in these repeats accounts for these low molecular weight fragments specifically in the ICF samples because of the virtual identity of the tandem copies of the 3.3 kb repeat at 4q35 (26,30).

Fibroblast DNA of one ICF patient (patient 3) was also analyzed by 2D gels and demonstrated the occurrence of fragments 1–5 due to demethylated *D4Z4* repeats as in B cells (data not shown). In contrast, normal individuals did not display *Eag*I *D4Z4* fragments in a variety of tissues including liver, ovary and brain. Similarly, DNA digests derived from malignancies of liver, ovary and brain tissues did not display *Eag*I *D4Z4* fragments.

The repeat unit of *D4Z4* contains two predicted homeobox domains. However, no transcript from this sequence has been reported from various tissues (28). No transcripts from these homeobox-like sequences were observed by RT–PCR in the B cell lines of ICF patients or their unaffected parents (data not shown).

Table 1. Summary of fragments with intensity changes in ICF patients' 2D patterns relative to those of their parents that are attributable to methylation changes

No. of patients affected	No. of fragments with increased intensity	No. of fragments with decreased intensity
1 of 4	44	52
2 of 2	19	4
3 of 4	14	1
4 of 4	8	5

2D patterns of individual patients showed that a minor fraction of the DNA fragments had appreciably altered intensities compared with those of their unaffected parents. Some of these changes occurred in all four patients. Changes observed in individual patients may represent random methylation events. Either increased or decreased intensity of a fragment may result from hypo- or hypermethylation.

Demethylation of the *NBL2* repeat and its overexpression in ICF syndrome

We previously cloned fragment 9 (Fig. 1) and identified it as a part of a tandem repeat sequence, which we designated *NBL2*, and which is abnormally hypomethylated in neuroblastomas at a high frequency (21). To confirm the demethylation of *NBL2* in ICF patients, Southern blotting was performed with a cloned PCR product of *NBL2* as a probe (Fig. 2B). Patients with the ICF syndrome, but not their phenotypically normal parents, exhibited four hybridizing low molecular weight bands (Fig. 2B) of approximately the same first-dimensional size as fragments 9–12 in the 2D gels (Fig. 1). These fragments are predicted from the *Eag*I restriction map of *NBL2*.

We tested whether *NBL2* sequences are transcribed specifically when they are demethylated by examining the expression of *NBL2* in B cell lines from patients and their unaffected parents. RT–PCR analysis was done on three of the families. Any contaminating genomic DNA in RNA samples was removed by treatment with DNase I, as confirmed in the amplification reaction without prior reverse transcription. *NBL2* RNA was present in the cell lines from all three patients at much higher steady-state levels than in the cell lines from their parents, as seen after 25 and 35 cycles of amplification (Fig. 3). That we saw similar differences in levels between patients and their parents, with extents of amplification that gave either low intensity bands or higher intensity bands, indicates that the results are not skewed by overamplification. In three other cell cultures with demethylated *NBL2* sequences, namely a fibroblast cell strain from ICF patient 3 and neuroblastoma cell lines SY5Y and SKNSH, we also observed expression of *NBL2* RNA by RT–PCR, whereas under the same conditions two normal B cell lines without demethylated *NBL2* sequences did not express detectable *NBL2* RNA (data not shown). These results suggest that demethylation of *NBL2* DNA repeats results in the up-regulation of their transcription in ICF patients.

DISCUSSION

The ICF syndrome is the first known disease with Mendelian inheritance of a DNA methylation deficiency (12–14). ICF recently has been shown to involve a defect in one of the genes

Table 2. Estimated copy number of affected fragments in 2D patterns of ICF families

Spot no.	Size (kb) (1D/2D)	Identity	Family 1			Family 2			Family 3		Family 4		
			P	F	M	P	F	M	P	M	P	F	M
1	2.2/0.74	*D4Z4*	50	2	8	50	0	0	20	2	50	0	2
2	2.4/0.74	*D4Z4*	2	2	2	8	0	0	8	2	10	2	2
3	2.4/0.33	*D4Z4*	2	2	2	8	0	0	8	2	10	2	2
4	3.1/0.26	*D4Z4*	4	0	0	4	0	0	4	0	4	0	0
5	3.3/0.59	*D4Z4*	8	1	1	4	2	2	2	0	4	0	2
6	1.8/0.50	?	10	0	0	10	2	0	10	1	10	2	2
7	1.8/0.32	?	10	0	0	10	0	0	10	0	10	0	0
8	3.6/2.0	?	2	0	0	2	0	0	2	0	2	0	0
9	1.4/0.19	*NBL2*	4	0	0	10	0	0	10	0	20	0	0
10	2.8/0.19	*NBL2*	2	0	0	2	0	0	8	0	10	0	0
11	4.2/0.19	*NBL2*	0	0	0	0	0	0	4	0	4	0	0
12	5.6/0.19	*NBL2*	0	0	0	0	0	0	2	0	2	0	0
13	4.2/0.70	?	0	2	2	0	4	4	0	4	0	4	4
14	3.0/0.44	?	0	4	10	2	10	8	1	10	2	10	8
15	2.4/0.34	?	0	2	2	0	2	4	0	2	0	2	4
16	5.5/0.35	?	0	2	2	0	8	8	0	4	0	8	8
17	5.5/0.30	?	2	10	4	0	8	10	2	10	0	8	10
18	3.0/0.97	VNTR	0	0	20	30	0	30	0	50	20	0	30

The intensities of the fragments were evaluated visually on 2D gels. The sizes of the fragments were estimated according to the size and location of known landmark fragments. The fragment numbers correspond to those in Figure 1: P, patient; F, father; M, mother. In all four patients, fragments 1 and 4–10 had increased intensities, and fragments 13–17 had decreased intensities. Fragment 18 is macrosatellite VNTR in Xq24. Identification was performed by sequencing of the cloned DNA fragments and Southern blotting.

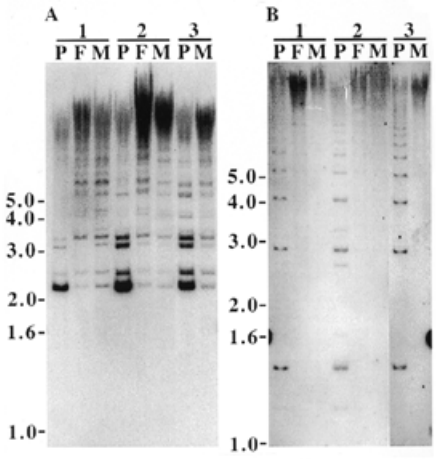

Figure 2. Analysis of methylation of (**A**) *D4Z4* and (**B**) *NBL2* in ICF patients and their unaffected parents by Southern blotting. A 5 µg aliquot of genomic DNA of B cells of the indicated subjects was digested with *Eag*I and separated in a 1% agarose gel. The DNA was transferred to a nylon membrane and hybridized with the appropriate probe. Three families (1, 2 and 3) were studied: P, patient; F, father; M, mother. The sizes of the bands in (A) correspond to those of fragments 1, 2, 3, 4 and 5 (2.2, 2.4, 2.4, 3.1 and 3.3 kb, respectively). The sizes of the bands in (B) correspond to those of fragments 9, 10, 11 and 12 in the first dimension of Figure 1 (1.4, 2.8, 4.2 and 5.6 kb, respectively).

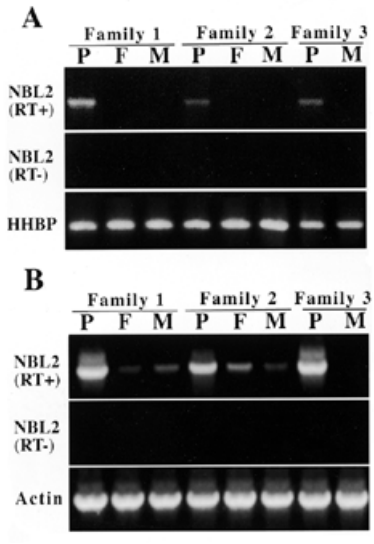

Figure 3. Analysis of *NBL2* RNA in the B cells of ICF patients and their parents by RT–PCR. Families 1, 2 and 3 were studied: P, patient; F, father; M, mother. To confirm the absence of contaminating genomic DNA by treatment with DNase I, the same RNA samples were amplified with (RT+) or without (RT–) reverse transcription. RT–PCR was performed for 25 (**A**) or 35 (**B**) cycles. The size of *NBL2* PCR products is 496 bp (A) and 838 bp (B). mRNAs of human 23 kDa highly basic protein (HHBP; PCR product 484 bp) and β-actin (PCR product 838 bp) were also amplified as a control. Note that *NBL2* RNA was present in the ICF patients at much higher levels than in their parents.

encoding DNA methyltransferase (*DNMT3B*) so that studying abnormal methylation of DNA in ICF gives insight not only into relationships between genomic undermethylation and development, but also into the DNA sequence specificity of DNMT3B *in vivo*. In this study, DNA methylation in the ICF syndrome was investigated for the first time by a method that examines a large fraction of the genome for ICF-specific hypomethylation, namely 2D electrophoresis of DNA digested with two restriction endonucleases, one of which (*Eag*I) is sensitive to CpG methylation (19). Despite the extreme rarity of this disease, we were able to compare digests from lymphoblastoid cell lines of four unrelated ICF patients with those of their parents to search for consistent ICF-associated differences in the intensity of DNA fragments, indicative of their undermethylation specifically in the patients. Most of the fragments in these digests had similar intensities in the samples from the patients and their parents. This is consistent with our recent findings that the 5-methylcytosine content of DNA from ICF cells is only a little less than that of normal cells. For example, ICF brain DNA has 7% less 5-methylcytosine than normal brain DNA (M. Ehrlich and E. Fiala, unpublished data). Therefore, most *Eag*I-containing CpG islands are resistant to the aberrant hypomethylation in the ICF syndrome associated with the deficiency of DNMT3B.

In contrast to most of the genome, two DNA repeats, *D4Z4* and *NBL2*, were strongly hypomethylated at the examined *Eag*I sites in ICF patients but not in their parents, as determined by analysis of fragments in 2D gels and Southern blotting. Therefore, in addition to satellite 2 and 3 DNA from the juxtacentromeric heterochromatin of chromosomes 1 and 16 (5–7,34), two non-satellite DNA repeats are undermethylated abnormally in all examined ICF samples. It is not just the high copy number of these sequences that confers on them their undermethylation in ICF cells. Many other highly repeated sequences that were digested by *Eag*I, as shown by their migration in the first dimension on the 2D gels, were approximately equally intense in the ICF patients' samples and their parents' samples (Fig. 1). For example, cloning and sequencing of fragment 18 revealed that this fragment is a part of a GC-rich macrosatellite variable number of tandem repeats (VNTR) in Xq24 (Table 2) (35). This sequence has been reported to be methylated on the active X chromosome and demethylated on the inactive X chromosome (35). 2D analysis showed that this fragment was methylated and was absent in 2D gels of male patients (patients 1 and 3) and fathers, but it had similar intensities in female patients (patients 2 and 4) and mothers. These results indicate a specificity of DNMT3B for certain DNA sequences or chromatin regions and inability of other DNA methyltransferases to compensate for the loss of DNMT3B *in vivo* in ICF patients, although, *in vitro*, the highly homologous mouse *Dnmt3b* shows no sequence specificity other than for CpG (36). The *D4Z4*, *NBL2*, satellite 2 and satellite 3 sequences do not share any obvious sequence motifs. The *NBL2* and *D4Z4* repeat units, unlike satellite DNA consensus sequences, do not consist of very small subrepeats.

We found that *NBL2* repeats were hypomethylated in normal sperm (data not shown) and in most examined neuroblastomas (21) as well as in ICF cells (Fig. 2B). Hypomethylation of repetitive sequences in cancers may lead to illegitimate chromosome recombination and resulting DNA gains and losses that promote oncogenesis (37). Furthermore, because the hypomethylation of *NBL2* repeats in ICF cells and neuroblastoma cells was accompanied by up-regulation of their transcription (Fig. 3), overexpression of these repeats might play a role in the ICF phenotype or in cancer formation. Identification of open reading frames (ORFs) in the *NBL2* sequence showed a short ORF encompassing two of the repeats, which did not have high homology with any known protein-encoding sequence. We searched for homology to previously identified coding sequences in the dbEST using Blastn and found several expressed sequence

tag (EST) clones with high homology to *NBL2* (data not shown). There is no typical polyadenylation signal within the *NBL2* sequence. However, it is possible that the *NBL2* transcript might be a non-coding but functional RNA, similar to *XIST* (38), *H19* (39) and antisense *IGF2R* RNA (40). Furthermore, aberrant methylation of *H19* and a loss of imprinting of *IGF2* have been observed in several types of tumor and inherited human genetic disease (reviewed in ref. 41). The lack of an increased incidence of cancer among ICF patients may be due to the small number of reported patients (<40) and the young age at which many die, precluding detection of long-term effects of localized DNA hypomethylation on cancer formation.

The hypomethylation of the sequence *D4Z4*, a tandem subtelomeric repeat at 4q35 and at 10q26, may help to elucidate the molecular basis for another rare genetic disease in addition to ICF. Although the muscular abnormalities of FSHD are not observed in ICF, neurological abnormalities often have been seen in a subset of patients with either syndrome (3,42). The DNA region altered in the dominantly inherited FSHD syndrome has been localized to the *D4Z4* repeats at 4q35. Normal individuals have ~12–100 copies of this repeat at this locus, but almost all studied FSHD patients have a reduction in the copy number of this repeat to <10 and there is a correlation between the severity of the disease and the number of deleted copies (33). Because the deletion encompasses only the repeat units, its reduction in copy number has been concluded to be responsible for FSHD, probably by position effect variegation affecting transcription of an unknown gene near a heterochromatic *D4Z4* repeat region at 4q35 (27) or possibly by harboring a gene sequence itself, which is expressed only in certain tissues of FSHD patients (28).

This is the initial report of the methylation status of the *D4Z4* repeats. We found that they were highly methylated in normal cells (the cells from the phenotypically normal parents of ICF patients), in contrast to their undermethylation in ICF cells. These findings may be related to effects of methylation on chromatin structure, just as hypomethylation of satellite 2 DNA sequences in chromosomes 1 and 16 of ICF cells is linked to abnormal decondensation of juxtacentromeric heterochromatin (3,6). Chromatin compaction, especially in heterochromatin, is, in turn, generally linked to transcriptional inactivation. *D4Z4* repeat units contain *LSau* subrepeats and are adjacent to 68 bp *Sau*3A repeats, both of which are associated with heterochromatic regions, including the juxtacentromeric heterochromatin of chromosome 1 (30,43,44). Part of the *D4Z4* repeat is 84% homologous to the repetitive sequence element hhspm3 (30), which we cloned on the basis of its hypomethylation specifically in sperm (45). As mentioned above, it has been proposed that some gene in the vicinity of the *D4Z4* repeats is abnormally expressed in FSHD as a consequence of the disease-related low copy number of *D4Z4* repeats (26). We hypothesize that such a gene might also be expressed, inappropriately although possibly with a different tissue specificity and at different levels, in ICF cells as a result of *D4Z4* hypomethylation.

MATERIALS AND METHODS

Subjects

EBV-transformed B cell lines from four ICF syndrome families consisting of four affected patients (patients 1–4) and seven unaffected parents were studied. The clinical and cytogenetic findings of patients 1 and 4 were reported previously (3,46). The abnormal methylation of α- and classical satellites of patients 2 and 3 was studied previously (10). Patients 1 and 3 are male. Patients 2 and 4 are female.

2D gel electrophoresis

Genomic DNA of B cells and fibroblasts was extracted using DNAzol (Gibco BRL, Gaithersburg, MD) according to the manufacturer's protocol. In addition, DNA was extracted from liver, ovarian and brain malignancies, as well as normal tissue-specific controls. The extracted DNA was separated by 2D gel electrophoresis as previously reported, with some modifications (24). Briefly, 2.7 µg of DNA was treated with DNA polymerase I in blocking buffer and digested with the methylation-sensitive restriction enzyme, *Eag*I. The sticky ends of the DNA fragments were labeled with [α-^{32}P]dGTP and [α-^{32}P]dCTP using Sequenase Version 2.0, then separated in a 0.9% agarose tubular gel at 100 V for 66 h. The portion of the gel containing DNA fragments measuring ~1–6 kb was excised and the DNA was digested further *in situ* with *Hin*fI. The agarose gels were run in the second dimension in a vertical 4.75% polyacrylamide gel at 100 V for 44 h. Gels were dried onto filter paper and exposed to phosphoimage plates (Molecular Dynamics, Sunnyvale, CA) for 2 days. The images were scanned and analyzed as described previously (19). In preparative gels used for cloning, 100 µg of DNA was digested and extracted with phenol. The DNA fragments were end-labeled as above and mixed with unlabeled DNA, then applied onto agarose gels as above. For the second dimension, *Dpn*II was used for *in situ* digestion. The 2D gels were placed on filter paper and exposed to X-ray film at –80°C for 5 days.

Cloning and sequencing of DNA fragments

The *Eag*I–*Dpn*II fragments of interest were recovered from the gels and subsequently cloned into a modified pBC vector (Stratagene, La Jolla, CA) as described previously (21).

Southern blotting

A 5 µg aliquot of DNA was digested with 50 U of *Eag*I for 20 h according to the manufacturer's protocol. Genomic DNA digests were separated on a 1.0% agarose gel, transferred to a nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ) and hybridized with [α-^{32}P]dCTP-labeled probe for 16 h. The cloned *D4Z4* fragments from the 2D gels and the cloned PCR product from the *NBL2* sequence were used as probes. After washing with SSC buffer, the membranes were subjected to phosphorimaging, as above, for 16 h.

RT–PCR analysis

Total RNA was extracted from B cells using standard reagents (TRIZol; Gibco BRL). A 1 µg aliquot of total RNA was treated with 3 U of DNase I at 37°C for 35 min to digest the contaminating genomic DNA, then incubated at 75°C for 5 min to inactivate the DNase I. Reverse transcription was performed in a 33 µl reaction volume with 200 U of Superscript II (Gibco BRL) and random primers (Boehringer Mannheim, Indianapolis, IN). Superscript II was omitted from the reaction for negative controls. PCR was performed on 1 µl of the reverse transcription reaction product in a 25 µl reaction volume using standard procedures

(Expand High Fidelity PCR system; Boehringer Mannheim). Cycling conditions were as follows: an initial denaturation step of 3 min at 93°C, followed by 61°C for 30 s, 71°C for 1 min, 93°C for 30 s. After 10 cycles, the annealing and extension temperatures were changed to 62 and 72°C, respectively, then another 15 or 25 cycles were performed. Final extension was done at 72°C for 10 min. The primers used for amplification for *NBL2* were either Primer Set 1 (5′-TGTTCGTCTTTGCAGTTGTCCT-3′ and 5′-TCCACTCCTGACAGATAGGCTG-3′) or Primer Set 2 (5′-GTTTGTACCAGCCGAAGTTGC-3′ and 5′-TCCACTCCTGACAGATAGGCTG-3′). As an internal control, all the mRNA preparations were subject to RT–PCR with primers for human 23 kDa highly basic protein or actin (Clontech, Palo Alto, CA).

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