An imprinted locus associated with transient neonatal diabetes mellitus

Rebecca J. Gardner¹, Deborah J.G. Mackay¹, Andrew J. Mungall¹, Constantin Polychronakos², Reiner Siebert³, Julian P.H. Shield⁴, I. Karen Temple⁵ and David O. Robinson⁶

Wessex Regional Genetics Laboratory, Salisbury District Hospital, Salisbury, Wiltshire SP2 8BJ, UK, ¹The Sanger Centre, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SA, UK, ²MTL, Children’s Hospital Research Institute, Room 236, 4060 St Catherine Street West, Westmount, Quebec, Canada H3Z 2Z3, ³Department of Human Genetics, University of Kiel, 24105 Kiel, Germany, ⁴Institute of Child Health, St Michael’s Hill, Bristol BS2 8BJ, UK and ⁵Wessex Clinical Genetics Service, The Princess Anne Hospital, Southampton SO16 5YA, UK

Received 22 October 1999; Revised and Accepted 5 January 2000

Recently, we reported the localization of a gene for transient neonatal diabetes mellitus (TNDM), a rare form of childhood diabetes mellitus, to an ∼5.4 Mb region of chromosome 6q24. We have also shown that TNDM is associated with both paternal uniparental disomy (UPD) of chromosome 6 and paternal duplications of the critical region. The sequencing of P1-derived artificial chromosome clones from within the region of interest has allowed us to further localize the gene and to investigate the methylation status of the region. The gene is now known to reside in a 300–400 kb region of 6q24 which contains several CpG islands. At one island we have demonstrated differential DNA methylation between patients with paternal UPD of chromosome 6 and normal controls. In addition, two patients with TNDM, in whom neither paternal UPD of chromosome 6 nor duplication of 6q24 have been found, show a DNA methylation pattern identical to that of patients with paternal UPD of chromosome 6. Control individuals show a hemizygous methylation pattern. These results show that TNDM can be associated with a methylation change and identify a novel methylation imprint on chromosome 6 associated with TNDM.

INTRODUCTION

Transient neonatal diabetes mellitus (TNDM) presents in growth retarded neonates with persistent hyperglycaemia within the first 6 weeks of life and has an incidence of ∼1 in 400,000 live births. Patients usually require exogenous insulin therapy for a mean duration of 3 months while endogenous insulin levels are either extremely low or undetectable. Recovery is complete by 18 months of age (1), although ∼40% of patients relapse and develop type 2 diabetes later in life (2). Clinical investigations have shown that patients with TNDM do not have islet cell antibodies or diabetes-susceptible HLA haplotypes (3). This suggests a form of diabetes with more similarities to type 2 diabetes than to the classic autoimmune-related type 1 diabetes.

We have described previously the association of both paternal uniparental isodisomy of chromosome 6 (UPD 6) and large paternal duplications of 6q24 with TNDM (4–6). These observations have been verified by others (7–10), although two cases of paternal UPD 6 are reported with no mention of TNDM (11,12). One case of maternal UPD 6 with no evidence of TNDM is also reported in the literature (13). These findings lead to the hypothesis that TNDM is caused by the overexpression of an imprinted gene which we have already localized to an ∼5.4 Mb region of 6q24 (14).

In order to localize the gene further we have adopted a quantitative PCR approach to identify patients with submicroscopic duplications within the 5.4 Mb region of interest. This, in combination with sequence for the region now being available as part of the Sanger Centre’s chromosome 6 sequencing project, has enabled us to look at a small region of the genome in more detail and identify landmarks such as previously characterized genes, putative genes and CpG islands.

As all previously described imprinted genes have been associated with parent of origin methylation differences, we investigated the methylation status of the CpG islands in our region of interest. The majority of CpG dinucleotides in the genome are methylated whereas those found in CpG islands are generally protected from methylation and are associated with housekeeping genes (15). The majority of imprinted genes have an inactive methylated allele and an unmethylated active allele, although the reverse has also been found, and are mono-allelically expressed (16). Our aim was to examine the methylation status of the CpG islands within the critical region by comparing the methylation of CG sequences within methylation-sensitive restriction enzyme sites in DNA from patients with paternal UPD 6 and normal controls. Once differential methylation was detected we analysed the methylation status of the CpG islands in our TNDM patients.

Using this combination of approaches, we have further defined the critical region and have identified parent of origin specific DNA methylation indicative of imprinting. In addition

¹These authors contributed equally to this work
²To whom correspondence should be addressed. Tel: +44 1722 336262 ext. 4080; Fax: +44 1722 338095; Email: drobinso@hgmp.mrc.ac.uk
we have analysed 13 patients in whom no aberration of chromosome 6 had been identified previously, and demonstrated the loss of methylation in two of them, confirming that this locus is almost certainly involved in the aetiology of TNDM.

RESULTS

Refinement of the region

A critical region of ∼5.4 Mb had already been identified from patients with large 6q duplications. To refine this region further we adopted a quantitative PCR approach for the detection of submicroscopic duplications. We screened TNDM patients who do not have UPD 6 or a visible duplication by amplifying sequence tagged sites (STSs) and expressed sequence tags (ESTs) within the critical region in duplex with a control primer set from a different chromosome. The dosage quotient was calculated by dividing the ratio of peak height between the chromosome 6 product and the control product by the same ratio from normal control individuals. Several individuals with known duplications were also tested to allow comparison of the dosage quotients between patients and known duplications (Fig. 1 and Table 1). In five families we identified submicroscopic duplications which were overlapping and of varying size (Fig. 2). In all cases where it was possible to determine parental origin, the duplication was paternal. Duplication A was found in the proband, affected brother and affected father of family A, but not in the mother or in several other paternal female relatives. Family B has been described previously in detail (5,14) and the duplication has been paternally inherited by all the affected individuals. Duplication C is found in the proband and father, duplication D in the proband, father and brother (who had type 2 diabetes) and duplication E in the proband only, as the parents were unavailable for testing.

The smallest region of overlap is between the duplications found in families A and B, is between 300 and 400 kb long and is contained within a mapped contig in the 6ace database at the Sanger Centre. An exact size cannot yet be determined as one gap in the sequence tiling path remains to be closed.

Structure of the region

The newly defined critical region is covered by the P1-derived artificial chromosomes (PACs) 468K18, 340H11, 197L1 and 91J24 from which sequence is available (courtesy of the Sanger Centre; accession nos AL049844, AL109755, AL031390 and AL024474, respectively) and 83M4 which is awaiting sequencing (by the Sanger Centre). The annotated sequence of these PACs contains six CpG islands, one complete characterized gene (PLAGL1/ZAC/LOT1) (17–19) and numerous ESTs, of which we have used 11 for PCR dosage studies (stSG24859, stSG1437, stSG8766, stSG9563, stWI-11634, stEST99515, stdJ218N6T7, stSG24869, stSG27894, stSG20934 and stSG11228). These features, as well as the integrated molecular analysis of genomes
and their expression (IMAGE) sequence 2073154, accession no. A1540783 (20), are shown in Figure 2.

**Methylation analysis**

In order to identify candidate genes we looked for features of imprinting. In particular we analysed CpG islands for the presence of methylation differences between maternal and paternal alleles using DNA from TNDM patients with paternal UPD 6 and normal controls.

*Hpa*II and *Msp*I restriction enzymes both cleave at CCGG sites. However, *Hpa*II will not cleave when the internal C is methylated. DNA was digested with these two enzymes independently, followed by amplification by PCR to test for the presence or absence of a PCR product. *Msp*I cuts regardless of methylation status and confirmed the presence of the CCGG site. A product is visible in the *Hpa*II lane only if digestion has occurred (i.e. the internal C is methylated). In order to make the DNA more accessible for digestion with *Hpa*II and *Msp*I the DNA was also digested with the restriction enzyme *Mse*I which has the recognition site TTAA and does not cleave within the sequence amplified.

The six CpG islands indicated in Figure 2 were analysed using this method. Due to the density of CCGG recognition sites it was not always possible to analyse each site individually so up to three sites were grouped in any one PCR amplification. For five of the CpG islands there was no amplification. For six of the CpG islands there was no amplification and the positions of the CpG islands (boxes). Black boxes show the islands that contain CCGG sites which are unmethylated in UPD 6 patients and controls; a white box shows a CCGG site which is fully methylated in UPD 6 patients and controls; and a grey box indicates the island in which the CCGG site is differentially methylated. The known gene *PLAGL1* is indicated by the dotted line. This figure is based on the data obtained from the Sanger Centre’s chromosome 6 mapping and sequencing projects.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Radiation hybrid map position (cR3000)</th>
<th>Duplication controls</th>
<th>Family B TNDM patients</th>
<th>Status*</th>
<th>Family B members without TNDM</th>
<th>Status*</th>
</tr>
</thead>
<tbody>
<tr>
<td>stSG24859</td>
<td>561.83</td>
<td>1.52 ± 0.11 (n = 11)</td>
<td>1.01 ± 0.09 (n = 9)</td>
<td>–</td>
<td>1.03 ± 0.10 (n = 8)</td>
<td>–</td>
</tr>
<tr>
<td>stSG8766</td>
<td>561.83</td>
<td>1.51 ± 0.19 (n = 12)</td>
<td>1.11 ± 0.16 (n = 10)</td>
<td>–</td>
<td>1.01 ± 0.11 (n = 8)</td>
<td>–</td>
</tr>
<tr>
<td>stSG1437</td>
<td>562.13</td>
<td>1.55 ± 0.32 (n = 16)</td>
<td>0.85 ± 0.09 (n = 16)</td>
<td>–</td>
<td>0.85 ± 0.23 (n = 6)</td>
<td>–</td>
</tr>
<tr>
<td>stSG9563</td>
<td>564.39</td>
<td>1.44 ± 0.20 (n = 16)</td>
<td>1.47 ± 0.22 (n = 20)</td>
<td>+</td>
<td>0.99 ± 0.09 (n = 10)</td>
<td>–</td>
</tr>
<tr>
<td>st-W111634</td>
<td>565.9</td>
<td>1.64 ± 0.28 (n = 9)</td>
<td>1.42 ± 0.15 (n = 5)</td>
<td>+</td>
<td>0.97 ± 0.13 (n = 6)</td>
<td>–</td>
</tr>
<tr>
<td>stEST99515</td>
<td>565.89</td>
<td>1.51 ± 0.18 (n = 17)</td>
<td>1.45 ± 0.18 (n = 18)</td>
<td>+</td>
<td>1.06 ± 0.12 (n = 9)</td>
<td>–</td>
</tr>
<tr>
<td>stdJ218N6T7</td>
<td>Not determined</td>
<td>1.43 ± 0.10 (n = 9)</td>
<td>1.32 ± 0.11 (n = 5)</td>
<td>+</td>
<td>0.91 ± 0.07 (n = 5)</td>
<td>–</td>
</tr>
<tr>
<td>stSG24869</td>
<td>567.8</td>
<td>1.64 ± 0.24 (n = 17)</td>
<td>1.38 ± 0.13 (n = 17)</td>
<td>+</td>
<td>0.94 ± 0.08 (n = 9)</td>
<td>–</td>
</tr>
<tr>
<td>stSG27894</td>
<td>567.8</td>
<td>1.44 ± 0.06 (n = 8)</td>
<td>1.48 ± 0.10 (n = 5)</td>
<td>+</td>
<td>1.00 ± 0.11 (n = 6)</td>
<td>–</td>
</tr>
<tr>
<td>stSG20934</td>
<td>567.8</td>
<td>1.55 ± 0.27 (n = 7)</td>
<td>1.03 ± 0.09 (n = 6)</td>
<td>–</td>
<td>1.12 ± 0.15 (n = 4)</td>
<td>–</td>
</tr>
<tr>
<td>stSG11228</td>
<td>569.62</td>
<td>1.47 ± 0.14 (n = 6)</td>
<td>0.97 ± 0.06 (n = 6)</td>
<td>–</td>
<td>1.03 ± 0.13 (n = 6)</td>
<td>–</td>
</tr>
</tbody>
</table>

Figure 2. Diagram of the TNDM critical region. This figure shows the order and overlap of the PACs, the position of the STSs and ESTs used for quantitative PCR (arrows; markers stSG24859, stSG20934 and stSG11228 are not shown on the figure as they are outside this contig; the order of stSG1437 and stSG8766 is reversed in this figure compared with Table 1 as the sequence order differs from the radiation hybrid map order), the extent of the five submicroscopic duplications (+, duplicated; –, not duplicated) and the positions of the CpG islands (boxes). Black boxes show the islands that contain CCGG sites which are unmethylated in UPD 6 patients and controls; a white box shows a CCGG site which is fully methylated in UPD 6 patients and controls; and a grey box indicates the island in which the CCGG sites are differentially methylated. The known gene *PLAGL1* is indicated by the dotted line. This figure is based on the data obtained from the Sanger Centre’s chromosome 6 mapping and sequencing projects.
sequence available from the Sanger Centre’s anonymous ftp site; accession no. AL109755) differential methylation was observed. At two sites (H11F and H11D2) within this CpG island in PAC 340H11 (Figs 2 and 3) there was no PCR product following digestion of the paternal UPD 6 DNA with HpaII/MseI whereas a product was observed after amplifying the HpaII/MseI-digested control DNA samples (Fig. 4a). This suggests that the paternal allele is unmethylated whereas the maternal allele is methylated.

We then analysed all TNDM patients who had neither paternal UPD 6 nor a duplication. This was achieved by using the above strategy but with quantitative PCR in duplex with a control set of
Paternal UPD 6. DNA was digested with DNA and lack of methylation in DNA from individuals with within the island that exhibit hemizygous methylation in control altered methylation pattern is associated with TNDM and is not digestion PCR product (data not shown). This confirms that the agarose gel and all showed the presence of the post-
PST in duplex with the methylation-sensitive enzymes EagI, NotI, SmaI and SacII, respectively (Figs 3 and 5). PstI cleaves either side of the CpG island. Within the PstI fragment lie one EagI site, two NotI sites, two SmaI and three SacII sites. The blot was probed with IMAGE clone 2073154 located within the PstI fragment and the results are shown in Figure 5. Digestion with PstI alone gives a single band of ~3300 bases with both normal and paternal UPD 6 DNA. In control DNA, EagI partially digests the PstI fragment resulting in two bands of approximately the same intensity, one the original PstI band and a second smaller band of ~2600 bases. In contrast EagI completely digests the PstI fragment in paternal UPD 6 DNA, resulting in only a single smaller fragment being visible on the Southern blot. This indicates that the EagI site is unmethylated in paternal UPD 6 DNA and hemimethylated in control DNA, being methylated only on the maternally inherited chromosome 6. The same pattern of bands was seen with NotI digestion, one of the NotI sites overlapping the EagI site and the second NotI site presumably being fully methylated on both chromosomes, not clearing in either normal or paternal UPD 6 DNA samples. Similarly, at least one of the SmaI and one of the SacII sites exhibited hemimethylation. The sizes of the fragments seen were in all cases consistent with the DNA sequence derived from PAC 340H11 by the Sanger Centre (accession no. AL109755).

**DISCUSSION**

We have shown previously that TNDM can result from paternal uniparental disomy (UPD) and from large paternally inherited duplications of chromosome 6 (4–6). This led us to theorize that TNDM is caused by overexpression of the paternal allele of an imprinted gene and that some non-UPD 6 patients with no obvious duplication may have small, submicroscopic duplications. We have now found five such cases which define a critical region of 300–400 kb in which the TNDM gene must lie.

**Table 2.** Results of the methylation analysis at sites F and D2 within PAC 340H11

<table>
<thead>
<tr>
<th>Type (no. of samples)</th>
<th>PCR F</th>
<th>PCR D2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of tests</td>
<td>Mean±SD</td>
</tr>
<tr>
<td>Normal controls (5)</td>
<td>13</td>
<td>0.50±0.037</td>
</tr>
<tr>
<td>Paternal UPD 6 (4)</td>
<td>11</td>
<td>0.00±0.000</td>
</tr>
<tr>
<td>Duplication of paternal origin (4)</td>
<td>15</td>
<td>0.39±0.075</td>
</tr>
<tr>
<td>Duplication of maternal origin (1)</td>
<td>4</td>
<td>0.62±0.070</td>
</tr>
<tr>
<td>Cause of TNDM unknown (11)</td>
<td>22</td>
<td>0.50±0.091</td>
</tr>
<tr>
<td>Methylation defect (2)</td>
<td>6</td>
<td>0.00±0.000</td>
</tr>
</tbody>
</table>

*Mean of the ratio between the HpaII/MseI-digest PCR:control PCR ratio and MseI-digest PCR:control PCR ratio.

Figure 5. Southern blot analysis of methylation sites in the differentially methylated CpG island by digestion of paternal UPD 6 DNA and normal control DNA with methylation sensitive restriction enzymes. P, DNA digested with PstI alone; P/E, DNA digested with PstI+EagI; P/N, DNA digested with PstI+NotI; P/M, DNA digested with PstI+SmaI; P/S, DNA digested with PstI+SacII.

We were also able to analyse individuals with paternally and maternally inherited duplications of the region. The observed ratios reflect the presence of two unmethylated alleles and one methylated allele when the duplication is paternally inherited, and two methylated alleles and one unmethylated allele when maternally inherited (Table 2).

In addition, DNA from 50 control individuals without TNDM was digested in the same way and amplified using primer sets H11F and H11D2. Each PCR was set up at least twice for each individual. Two of these patients showed a methylation pattern identical to that seen with the paternal UPD 6 samples whereas the remaining 11 showed hemizygous methylation, indicated by a ratio of 0.5 rather than 1 (complete methylation) or 0 (complete unmethylation) (Fig. 4b and Table 2).

Southern blot analysis was carried out to identify further sites within the island that exhibit hemizygous methylation in control DNA and lack of methylation in DNA from individuals with paternal UPD 6. DNA was digested with PstI alone and with PstI in duplex with the methylation-sensitive enzymes EagI, NotI, SmaI and SacII, respectively (Figs 3 and 5). PstI cleaves either side of the CpG island. Within the PstI fragment lie one EagI site, two NotI sites, two SmaI and three SacII sites. The blot was probed with IMAGE clone 2073154 located within the PstI fragment and the results are shown in Figure 5. Digestion with PstI alone gives a single band of ~3300 bases with both normal and paternal UPD 6 DNA. In control DNA, EagI partially digests the PstI fragment resulting in two bands of approximately the same intensity, one the original PstI band and a second smaller band of ~2600 bases. In contrast EagI completely digests the PstI fragment in paternal UPD 6 DNA, resulting in only a single smaller fragment being visible on the Southern blot. This indicates that the EagI site is unmethylated in paternal UPD 6 DNA and hemimethylated in control DNA, being methylated only on the maternally inherited chromosome 6. The same pattern of bands was seen with NotI digestion, one of the NotI sites overlapping the EagI site and the second NotI site presumably being fully methylated on both chromosomes, not cleaving in either normal or paternal UPD 6 DNA samples. Similarly, at least one of the SmaI and one of the SacII sites exhibited hemimethylation. The sizes of the fragments seen were in all cases consistent with the DNA sequence derived from PAC 340H11 by the Sanger Centre (accession no. AL109755).
The finding of a differentially methylated CpG island is indicative of an imprinted gene and the occurrence of methylation mutations at this locus in two patients with TNDM and not in 50 control individuals confirms its involvement in TNDM. There are at least two other possible explanations for these findings. An individual with a maternal deletion would have a methylation pattern identical to that of the paternal UPD 6 patients when viewed on an agarose gel. If this were the case the methylation ratio would be altered as the PCR following digestion with MseI alone would only be amplifying one copy (Table 2). This has not been observed and in addition, the quantitative PCR analysis of the critical region has not given any ratios which are indicative of a deletion rather than a duplication. Gene conversion is another possibility. However, both methylation mutation patients show heterozygosity of the adjacent polymorphic marker D6S310 (data not shown).

Our observations provide a mechanism for the cause of TNDM in two patients without paternal UPD or a paternal duplication of 6q. This leaves 11 TNDM patients in whom neither an aberration of chromosome 6 nor a methylation mutation could be identified. This is not surprising given the numerous mechanisms causing other disorders associated with imprinting, e.g. Beckwith–Wiedemann syndrome (21). Overexpression could be caused by the up-regulation of a gene due to an enhancer or regulatory sequence mutation. It is also possible that some patients have a very small duplication covering a single gene or regulatory region, though extensive coverage of the critical region by quantitative PCR analysis has not revealed such a duplication.

Although on some chromosomes imprinted genes are known to cluster (e.g. in the Beckwith–Wiedemann region on chromosome 11 and the Prader–Willi/Angelman syndrome region on chromosome 15) (22), we were able to identify differential methylation in only one CpG island in the critical region. It is possible that other genes on chromosome 6 in the critical region are imprinted and may be differentially methylated at sites other than those that we have investigated. Apart from TNDM, patients with paternal UPD 6 have no other clinical features, although macroGLOSSIA and umbilical hernia have been reported occasionally (I.K. Temple et al., submitted for publication). The relatively mild nature of the TNDM phenotype suggests that there may be few imprinted genes on chromosome 6. IGF2R, which is located ∼44 cR3000 telomeric to the imprinted CpG island in the TNDM critical region, exhibits methylation differences with evidence that it may be subject to polymorphic imprinting (23). Imprinted genes are reported to have several distinguishing features. It has been suggested that they have few and small introns (24) and are associated with direct repeats, though the repeats themselves are unique to each gene (25). They are differentially methylated, leading to monoallelic expression (16), and potential imprinting centres have been identified in some cases, although there is no consensus as to what defines an imprinting centre (26,27).

As it is duplication or overexpression rather than deletion of a gene that causes TNDM, candidate genes cannot be analysed by the standard mutation detection methods in the hope of identifying frameshift or stop mutations. Therefore, we will look for monoallelic expression of all candidate genes. To do this a transcribed polymorphism must be identified within the gene of interest, and an expressing tissue identified. As TNDM affects neonates who are growth-retarded at birth, it is probable that the gene has a role in development and it may be necessary to determine the stage of development during which the gene is expressed. These factors may make the identification of monoallelic expression of such a gene difficult. An example of this is UBE3A, the gene for Angelman syndrome. Monoallelic expression has only been detected in the brain whereas there is biallelic expression in other tissues (28,29). There is also the possibility that, as has been suggested for IGF2R, the gene for TNDM may be polymorphically imprinted (23). Therefore, despite the finding of a differentially methylated locus for TNDM, it may be some time before a monoallelically expressed gene is identified.

Our minimal duplicated region contains numerous ESTs but our search is confined to genes lying entirely within it and, more specifically, to those lying closer to the imprinted CpG island than to its unimprinted neighbours. One strong candidate is the IMAGE clone 2073154, accession no. AI540783 (20), which partially overlaps the island. Another candidate is PLAGL1, accession no. NM002656 (17), which has also been described as ZAC, accession no. AI006534 (18), and LOT1, accession no. U72621 (19), and is CpG rich and highly methylated in both control and UPD 6 individuals. It lies 85 kb from the imprinted CpG island, but may nonetheless be subject to imprint control.

In conclusion, we have further narrowed the TNDM critical region from 5.4 Mb to 300–400 kb and have identified parent of origin specific methylation differences in a CpG island within this region. Furthermore, we have found a loss of methylation in two TNDM patients, demonstrating that methylation at this locus is involved in the aetiopathogenesis of transient neonatal diabetes mellitus.

MATERIALS AND METHODS

Patients

All patients included in the study were referred to us with a diagnosis of TNDM and presented with persistent hyperglycaemia within the first 6 weeks of life (in pregnancies of >35 weeks gestation) and recovered by 18 months. Our control population was selected anonymously from samples already collected and stored within the department. DNA was extracted from whole blood using a salt precipitation method (30). Patients were first tested for UPD 6 followed by duplication analysis if they did not have UPD 6. Methylation analysis was only carried out on patients who did not have UPD 6 or a duplication.

Mapping and sequence information

Mapping information was obtained from the Sanger Centre’s chromosome 6 mapping database via the internet (http://www.sanger.ac.uk/HGP/Chr6r ). Sequence information was obtained from the Sanger Centre’s anonymous ftp site (ftp://ftp.sanger.ac.uk/pub/human/sequences/Chr_6/ ). All PACs were isolated from a library kindly donated to the Sanger Centre by Pieter de Jonge (http://bacpac.med.buffalo.edu ).

Primer design and manufacture

Primers were either ordered according to the sequences given in the Sanger Centre’s chromosome 6 database or designed in-house from the available sequence. Primers were manufactured...
by either MWG Biotech (Milton Keynes, UK) or Interactiva Biotechnology (Ulm, Germany).

**Duplication detection by quantitative PCR**

A 100 ng aliquot of DNA was amplified in duplex by a set of primers from chromosome 6 and a control set of primers for Dp2.54 on chromosome 5 (31). The chromosome 6 primers were: stSG24859, stSG1437, stSG8766, stSG9563, stWI-11634, stEST99515, std218N677, stSG24869, stSG27894, stSG20934 and stSG11228. One primer from each pair was labelled with a fluorophore during manufacture. The DNA was amplified using Hotstar Taq (Qiagen, Crawley, UK) as recommended by the manufacturer with additional MgCl2 to a final concentration of 2 mM. The concentrations of the primers were varied in order to optimize the duplex PCR. Amplification was in a Perkin Elmer (Warrington, UK) 9600 programmable heating block using the following conditions: 95°C for 15 min followed by 19 cycles (to keep the amplification in the exponential phase) of 94°C for 45 s; 52–58°C for 45 s (depending on the annealing temperatures of the individual primer sets); 72°C for 1 min followed by 7 min at 72°C and 1 h at 60°C. PCR products were run on an ABI 377 automated sequencer for separation and visualization.

For each PCR and gel a set of controls with and without known duplications of 6q was included. The ratio of the peak height between the chromosome 6 product and the control product was calculated and the dosage quotient determined by dividing the patient ratio by the average ratio obtained from the normal control samples.

**PCR amplification of sites showing parent of origin specific methylation differences**

The initial analysis to determine whether a site was differentially methylated was carried out on DNA samples from TNDM patients with paternal UPD 6 and normal controls. These digests were carried out per sample. DNA (2 µg) was digested overnight at 37°C with 3 U of *Hpa*II and *Mse*I, of *Msp*I and *Mse*I and of *Mse*I alone in a total volume of 20 µl. The following morning an additional 3 U of each enzyme was added and incubated for a further hour. They were then diluted to a final concentration of 25 ng/µl. The samples were amplified by PCR using primer sets H11F and H11D2 to analyse the CpG island lying at nucleotides 53299–53316 and 53179–53199. The primer sequences were H11D2, TGTGGGTGCCGCT-

The analysis within each lane was also the same as for the quantitative PCR. To calculate the degree of methylation, the ratios of the *Hpa*II/*Mse*I-digested samples were compared with the ratio obtained following amplification of the sample digested with *Mse*I alone. The 50 control samples analysed were digested and amplified in the same way as the initial paternal UPD 6 and control samples and visualized on 2% agarose gels.

**Southern blot analysis to show parent of origin specific methylation differences**

Southern blot analysis was carried out using standard techniques (32). Aliquots (7 µg) of normal control DNA and DNA from paternal UPD 6 individuals were digested with *Pst*I, *Pst*I + *Eag*I, *Pst*I + *Nor*I, *Pst*I + *Sma*I and *Pst*I + *Sac*II. The blot was probed with IMAGE clone 2073154 (20) (Fig. 3).

**ACKNOWLEDGEMENTS**

We would like to thank the chromosome 6 mapping and sequencing teams at the Sanger Centre for the genome information (http://www.sanger.ac.uk/HGP/Chr6/ ). We are extremely grateful to Drs R. Santer, D. Carson, M. Gonthier, R. Grabs-Palma, A. Fergusson, S. Wilson, V. Datta, G. Valerio, A. Franzese and P. McNally and all of the referring clinicians for sending patient samples to us. We also thank Professor Patrica Jacobs and Mr John Barber for their help and encouragement. R.J.G. and D.J.G.M. are supported by a grant from the British Diabetic Association and work at the Sanger Centre is supported by the Wellcome Trust.

**REFERENCES**


