The right end of transposable bacteriophage D108 contains a 520 base pair protein-encoding sequence not present in bacteriophage Mu

George B.Szatmari¹, Martine Lapointe² and Michael S.DuBow*

Department of Microbiology and Immunology, McGill University, 3775 University Street, Montreal, Quebec H3A 2B4, Canada

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ABSTRACT

We have cloned and characterized the right end terminal 796 bp of the transposable Mu-like bacteriophage D108. This region encompasses a 520 bp region of D108-specific sequences not present in phage Mu that contain an open reading frame encoding a 12 KDa protein. This protein can be visualized *in vivo* when the region is placed downstream from the strong *lac* UV5 promoter. The open reading frame can be expressed from the *dam*-regulated *mod* promoter (for modification of D108 DNA), yet also contains its own *dam*-independent promoter for expression that is detectable by northern blot analysis late in the D108 lytic cycle. Comparison of this region of D108 DNA with the corresponding region of Mu DNA suggests that a complex rearrangement has occurred at the phages' right ends during their evolution.

INTRODUCTION

Bacteriophage D108 is a transposable Mu-like temperate phage which can grow in *Escherichia coli* and many other enteric bacteria. D108 was first isolated by Mise (1) as a generalized transducing phage and was later shown by Hull *et al.* (2) to be a mutator phage. D108, like its close relative Mu, utilizes transposition to replicate and integrate its genome into its host's chromosomal DNA. An electron microscopic heteroduplex analysis (3) between Mu and D108 DNA showed the genomes of the phages were over 90% homologous. Four areas of non-homology were characterized; three occurring within the leftmost 9 kb, and one occurring at the extreme right end, also known as the β region of the phages. The largest region of non-homology resides about 250 - 1500 base pairs from the left end of the phages and accounts for different *c* (repressor) and *ner* genes (4,5) and encodes a different amino terminus for the *A* (transposase) gene (6). Because of these differences, the two phages are heteroimmune. However, despite the differences in their transposase genes, the gene products and their *cis*-acting DNA sequences required for transposition are virtually interchangeable, though at different efficiencies (7).

The region of non-homology at the extreme right end (β -region) of D108 was shown to contain an additional 500 bp of DNA occurring about 100 bp from the end of the phages, as revealed by electron microscopic heteroduplex analysis (3).

The β region of the phages is quite small, and few genes are found here. The first gene found in the β region, the *gin* gene, is involved in inverting a segment of DNA known as the G loop to allow the phages to alter their host ranges by encoding a different set of tail fibers (8). The remaining genes in this region include the *mom* gene of Mu, which is responsible for modifying Mu DNA to protect it from host restriction (9), and the *com* gene, a *mom*-specific regulatory protein (36). Toussaint *et al.* (6) showed that D108 has a DNA modification function similar to that of Mu. The *mom* gene of Mu ends 117 bp from the right end of the phage genome (10,11), and the Mu transposase has been found to bind to three apparent sites in this 117 bp region (7). The right end of D108 also displays a high degree of homology with the right end of Mu, but this homology was shown to end 72 bp from the right end of the phage (12).

In order to further characterize this region, we have cloned and sequenced 796 bp of the right end of bacteriophage D108 DNA. Analysis of the entire sequence shows that D108 has a modification gene which is similar, but not identical, to the *mom* gene of Mu. Because of this difference we have named this gene the *mod* gene, for *modification* of D108 DNA. The sequenced region also includes a 520 bp DNA sequence not present in the bacteriophage Mu β region. This insertion does not contain any of the common attributes of a mobile genetic element, and it has been found to encode a D108-specific gene product whose transcription is detectable late in the lytic cycle from its own *dam*-independent promoter and from the *dam*-dependent *mod* promoter. Moreover, the sequence differences between the extreme right ends of Mu and D108 indicate a complex evolutionary relationship through several blocks of nucleotides.

MATERIALS AND METHODS

Restriction endonuclease hydrolysis conditions, DNA ligations, and transformation of competent *E. coli* cells have all been described in detail elsewhere (5).

Bacterial Strains

The bacterial strains used in this study are all derivatives of *E. coli* K12. Strain JM103 (Δ *pro-lac*, *thi*, *rpsL*, *supE*, *endE*, *sbcB*, *hsdR*, *F'**traD36*, *proAB*, *lacI*^q*ZAM15*) was used as a recipient in

all transformations and for expression of cloned genes under *lac* control. Strains BU5029 (*Δpro-lac*, *thi*, *trp*, *rpsL*, *recA56*), LF107 (*Δpro-lac*, *thi*, *trp*, *rpsL*, *recA56*, *xyI::Mucts62*), LF4013 (*Δpro-lac*, *thi*, *trp*, *rpsL*, *recA56*, *mal::D108cts10*) were all used as *dam*⁺ hosts for RNA extractions. Strains GM48 (F⁺, *dam-3*, *dcm-6*, *thr-1*, *leu-6*, *thi-1*, *lacY1*, *galK2*, *galT22*, *ara-14*, *tonA31*, *tsx-78*, *supE44*), LF4044 (D108cts10 lysogen of GM48, prophage in unknown location), LF4045 (Mucts62 lysogen of GM48, prophage in unknown location), were used as *dam*⁻ hosts for RNA extractions. Strains MC4100 (F⁻, *araD139*, *ΔlacU169*, *rpsL*) and MC4100(PiCm) were used as indicators of *mom* or *mod* activity as conferring resistance to *Eco* P1 restriction (16). Strains LF4028 (*Δpro-lac*, *trp*, *rpsE*, *rpsL*, *D108^r* F'*pro-lacI::D108cts10*), and HM6305 (*Δpro-lac*, *his*, *met*, *rpsL*, *Mu^r*, F'*pro-lacZ6305::Mucts62*) were used as sources of D108cts10 and Mucts62 phage, respectively.

Media and growth conditions

Bacteria were routinely grown at 32°C in LB media (13) supplemented with 25 μg/ml ampicillin, when required. D108cts10 and Mucts62 phage were purified from strains LF4028 and HM6305, respectively, using the procedure of Ljungquist and Bukhari (14).

Testing of *mom* and *dam* phenotypes

The *mom* or *mod* phenotype of the phages produced after the Mu or D108 lytic cycle, respectively, were verified by plating dilutions of the phage lysate on strains MC4100 and MC4100(PiCm) according to the procedure of Toussaint (9). The *dam* phenotype of strains was tested by isolating the chromosomal DNA (14) and hydrolysing it with the restriction enzyme isoschizomers *Dpn* I, which only cleaves methylated DNA at the site 5' GATC 3' (15), or *Sau* 3AI, which cleaves at the same DNA sequence irrespective of its state of methylation, followed by electrophoresis on 1% horizontal agarose gels. The DNA in the gels was visualized by staining in 0.5 μg/ml ethidium bromide, followed by 260 nm ultraviolet illumination and photography on Polaroid type 57 film. DNA from *dam*⁻ *E. coli* was resistant to hydrolysis by *Dpn* I but not *Sau* 3AI, while DNA from *dam*⁺ hosts was cleaved by both enzymes.

DNA Sequence Analysis

The sequencing of the D108 right end *Alu* I fragment was performed according to the procedure of Maxam and Gilbert (16), with both strands sequenced in an overlapping manner. The sequence was checked for accuracy

by the identification of predicted restriction endonuclease sites and fragment lengths. For sequencing the D108 *mod* promoter, a D108 right end *Ava* I-*Sma* I fragment containing the terminal 2 kb of D108 and 200 bp of adjacent pSC101 sequences from pSZ53 (12), a pSC101 plasmid containing an entire D108 ϕ ts10 prophage, was used. This fragment was subcloned as a 400 bp *Hind* II-*Alu* I restriction fragment into the *Sma* I site of pUC119, a pUC19 derivative containing the M13 packaging site. Strains containing this plasmid were infected with M13K07 phage (Bio-Rad, Richmond, CA), and grown overnight in SB medium (17) containing 70 μ g/ml kanamycin and 150 μ g/ml ampicillin. Phages were isolated by PEG precipitation, extracted once with phenol, and the single-stranded DNA was precipitated with 0.3 M sodium acetate and 2 volumes of 100 % ethanol. This DNA was used directly for dideoxy sequencing (18) using the Bio-Rad M13 Klenow sequencing kit. Deoxyadenosine 5' [α -thio] triphosphate, [35 S] (500 Ci/mmol) was obtained from Dupont.

Cloning and Expression of the D108 right end terminal gene

An 816 bp *Alu* I fragment was isolated from plasmid pSZ5226, a mini-D108 derivative of plasmid pSZ52 (12). This fragment was then subcloned into the *Sma* I site of the pOP95-15K *lac* UV5 expression vector (5). The resultant plasmids were named pSZ21, for the plasmid with the D108 right end proximal to the *lac* promoter, and pSZ23, for the plasmid with the right end distal to the promoter.

The plasmid-encoded polypeptides were labelled with [35 S]-methionine (1120 Ci/mmol, Amersham) using the chloramphenicol release procedure of Neidhart *et al.* (19) and separated by SDS-polyacrylamide gel electrophoresis on 12.5% slab gels (20). The gels were prepared for treatment for fluorography by treatment with Amplify (Amersham) according to the manufacturer's instructions, dried, and exposed to Kodak XAR-5 X-ray film at -70°C.

Transcription of Mu and D108 right end genes

RNAs from induced *dam*⁺ and *dam*⁻ Mu and D108 lysogens were extracted (21), denatured in glyoxal (22), and subjected to electrophoresis on 1.5 % vertical agarose gels in 10 mM phosphate buffer, pH 6.5. The electrophoresis buffer was recirculated with a peristaltic pump. Transfer of RNAs to nitrocellulose (Schleicher and Schuell BA85) was performed using the procedure of Thomas (23), or by using the bidirectional transfer method of Smith and Summers (24), with 20 X SSC as the transfer buffer. After transfer, the blots were rinsed in 20 X SSC, air dried, and baked at

80°C under vacuum. The glyoxal adduct was denatured by boiling the filters in 20 mM Tris-HCl, pH 8 for 5 minutes. The filters were prehybridized overnight and hybridized according to Wahl *et al.* (25), and exposed to Agfa RPI X-ray film using Dupont Cronex intensifying screens. Radioactive probes were prepared by nick translation (26).

RESULTS

Sequence analysis of the right end of D108

The right ends of mature Mu or D108 phage DNA are notoriously difficult to characterize because of the variability of the 1 to 3 kb of *E. coli* DNA covalently attached as a result of headful packaging of dispersed prophages during lytic growth (27,28). In order to circumvent this problem, we utilized a mini-D108 plasmid which was derived from a D108 prophage insertion into the plasmid pSC101. In this case, the right end of the D108 prophage in plasmid pSZ52 is free from the variable *E. coli* DNA sequences and is conveniently located 20 bp from an *Alu* I site in pSC101 (12). A mini-D108 derivative of this plasmid was constructed by deleting 26 kb of internal D108 sequences using *Pst* I. The resultant plasmid, pSZ5226, has 1.7 kb of the left end and 8.0 kb of the right end, and is fully capable of transposition when provided with helper functions from an induced D108cts10 prophage (12). An *Alu* I site was mapped about 800 bp from the right end of D108 in this plasmid. This approximately 820 bp restriction fragment served as the substrate for the sequencing of the D108 right end. Moreover, we also cloned the 400 bp *mod* regulatory region into pUC119 in order to determine its sequence and compare it with that of the Mu *mom* regulatory region.

Comparison of the right end sequences of Mu and D108 DNA

The complete nucleotide sequence of both strands of the terminal 796 bp of the right end of phage D108 and the *mod* regulatory region was determined (Fig. 1). Comparison of the sequence obtained with that of phage Mu (10,11) reveals that D108 harbors an additional 520 bp of DNA beginning at nucleotide 73 from the right end. This additional DNA sequence begins at the next nucleotide after the third consensus transposase binding site (R3) in the D108 right end (12). The large open reading frame corresponding to the carboxy terminus of the *mod* gene, the D108 equivalent of *mom*, shows a high degree of homology with the *mom* gene of phage Mu. However, the *mod* gene terminates with a TAA codon at nucleotide (nt) 606 while *mom* terminates with a TGA codon at nt 121. This results in

Fig. 1. Nucleotide sequence of the right-end region of D108. (A) *mod* regulatory region: the *dam* sites involved in the regulation of the *mod* promoter are denoted by boxes and designated I, II and III (34) (B) The terminal 796 bp of the D108 right end. The boxed region indicates the 520 bp insertion. Putative promoter sequences are indicated (-35, -10), as well as the ribosome binding site (S.D.). Differences with the Mu sequence are indicated with the corresponding base of Mu placed above the D108 sequence. Changes which alter the predicted amino acid sequence are indicated by placing the altered amino acid of Mu beneath the indicated sequence. Sites R1, R2 and R3 refer to the putative transposase binding sites (7,12).

Fig 2. Organization of the Mu and D108 right ends. The thick lines indicate regions of DNA found in one phage but not the other. The large open reading frame is indicated above the insertion, starting with GTG and terminating with TGA.

mod terminating 3 codons before *mom*. Despite this difference, D108 still encodes a functional *mod* gene product (6). The *gin* gene of D108 was also found to be four amino acids longer than Mu's *gin* at the carboxy terminus. The 520 bp additional DNA shows no homology to any sequenced insertion element or transposon. No inverted repeats are found at the extremities of the additional DNA, although it is interesting to note that the sequence 5' TCT 3' is duplicated at the ends of this sequence. Further comparison of the right ends of D108 and Mu show that this additional insertion of DNA in D108 (or loss of DNA in Mu) is not a simple event (Fig. 2). The region of DNA between nt 73 and 120 in the right end of Mu is not found in D108 except for nt 88 to 99 which is found at position 593-602 from the right end of D108. The rest of the region in Mu is replaced by the insertion.

Finally, the 520 bp additional sequence reveals a large open reading frame (ORF) which could potentially encode a 102 amino acid polypeptide with a predicted molecular weight of 10.9 kilodaltons. A weak Shine-Dalgarno (29) sequence about 12 bp from the initiator GTG codon and potential -10 and -35 sequences (30) suggest that this ORF may be transcribed and translated, but not at a high efficiency.

The 520 base pair insertion can encode a 12 kilodalton polypeptide

In order to determine if this large ORF could be expressed *in vivo*, the terminal 800 bp of the D108 right end were cloned in the *lac* UV5 expression vector POP95-15K, as described in Materials and Methods. Plasmid pSZ21 has the ORF facing away from the *lac* UV5 promoter, while plasmid

Fig 3. (top) Restriction map of D108 right-end plasmids containing the 817 bp *Alu* I fragment from pSZ5226 cloned in pOP95-15K (as described in Materials and Methods). (bottom) 12.5% SDS-polyacrylamide gel showing crude extracts of ^{35}S -methionine-labelled chloramphenicol treated *E. coli* JM103 cells containing the indicated plasmids. Lanes 1-2 are 30 and 60 minute incubations of cells containing pSZ21 with ^{35}S -methionine after removal of chloramphenicol, lanes 3-5 are 10, 30 and 60 minute incubations of pSZ23-containing cells.

pSZ23 has the ORF placed downstream from the promoter (Fig. 3). When a chloramphenicol release assay (19) was performed on *E. coli* strains containing these plasmids, followed by SDS-polyacrylamide gel electrophoresis and fluorography, a polypeptide with an apparent molecular weight of 12 kilodaltons (kDa) was observed in strains carrying plasmid pSZ23. This 12 kDa polypeptide was also observed in pSZ21-containing cells, but in much smaller amounts.

Characterization of transcripts produced from the right end of Mu and D108

Using northern blotting and hybridizations, two transcripts were found to hybridize to the ORF-specific *Nde* I-*Eco* RI probe late in the D108 lytic cycle (Fig. 4). Both transcripts were produced 30 minutes into the D108 lytic cycle, and were not observable at the earlier time points. The 1500 nt transcript was observed to hybridize a *mod*-specific *Hinf* I probe, as well as the ORF-specific probe. This transcript was not seen at

Fig 4. Northern blot analysis of RNA extracted from D108 or Mu lysogens. A diagram of the D108 right end is shown on top, and the bold lines below this map indicate the probes which were used for northern blot hybridizations. The corresponding autoradiogram is shown below its respective probe. Numbers above the lanes indicate times, after thermoinduction, of the various D108cts10 or Muets62 lysogens from which the RNA was removed. Five micrograms of RNA were loaded in each lane. The autoradiogram on the left represents a 1.0% agarose gel blotted onto nitrocellulose. On this particular gel, in order to accurately determine the size difference between the transcripts, the 500 nt insertion-specific transcript was run off the gel. The autoradiogram on the right is a blot of a 1.5% agarose gel. On this gel, m refers to the BRL RNA ladder used as a molecular weight marker. The indicated molecular weights were estimated using the 1350 and 330 nucleotide RNA standards.

any time in *dam*⁻ induced Mu or D108 lysogens. The 950 nt Mu *mom* transcript (11,21), only seen late in *dam*⁺ induced Mu lysogens, hybridized with the *mod*-specific probe, but did not hybridize with the ORF-specific probe. A second transcript of about 500 nt was observed in induced D108 lysogens hybridizing to the ORF-specific probe. This transcript was seen in *dam*⁺ lysogens and at low, but detectable levels in *dam*⁻ D108 lysogens 30 minutes into the lytic cycle, but was not observed in any induced Mu lysogen. Both Mu and D108 growth and transcription were identical in these different genetic backgrounds, based on nor-

thern hybridizations of these lysogens probed with total Mu DNA, or a clone of the Mu late gene, *L* (not shown).

DISCUSSION

We have sequenced 796 bp of the right end of the bacteriophage D108 genome and have identified a 520 bp additional DNA sequence that begins 73 bp from the right end of the phage. This insertion does not appear to be the result of a transposition event into the D108 right end since the insertion does not possess any of the classical features attributed to transposons (31) with the exception of the directly repeated sequence 5' TCT 3' found at the ends of the insertion. The additional sequence appears to be the end result of a complex rearrangement which may have occurred during the evolution of Mu and D108. This rearrangement involved the deletion of nt 100-119 at the right end of Mu and the replacement of nt 73-83 with the 520 bp insertion. These changes do not seem to have disrupted the function of the *mod* gene, which, for the most part, is similar to Mu's *mom* gene though there are amino acid differences which could alter the charge of the protein (a his-ala change from nt 748, and a gly-glu change from nt 742). We also observed some subtle differences between the carboxy-termini of the *gin* genes, but this is not surprising, since the DNA invertases *gin*, *pin*, *cin* and *hin* all show some divergence at the carboxyl end (32). Homology between the two phages is restored 72 bp from the *attR* sites, which confirms that these sequences are important for the efficient transposition of these phages. Previous work by Craigie *et al.* (7) showed the existence of 3 potential binding sites for Mu's transposase at each end of the phage genome: L1-L3 at the left end and R1-R3 at the right end. The third putative binding site for transposase at the right end, R3, ends exactly 72 base pairs from the right end of the Mu genome. Groenen *et al.* (33) showed that the R3 site is not essential for mini-Mu transposition, though mini-Mu's which contain an intact R3 site have a slightly higher transposition frequency. While this site may be dispensable for mini-Mu (or mini-D108) DNA transposition, it may be required by the entire phage genome to transpose efficiently during replicative transposition in its lytic cycle. It is worth noting that Craigie *et al.* (7) demonstrated that the binding of purified Mu transposase to the Mu right end was strongest at this site, when compared to sites R1 and R2.

Bacteriophage Mu has been shown to produce a *dam*-regulated transcript of about 950-1100 nt in length (11,21). The 5' end of this mRNA has

been mapped to a position 973 bp from the *attR* site of Mu (11). Using northern blotting and hybridizations, we identified two transcripts being produced from this region of D108 late in the lytic cycle. A 1500 nt transcript was found to hybridize both a *mod*-specific probe and a 520 bp insertion-specific probe. The 1500 nt transcript is only observed in induced *dam*⁺ lysogens of D108. This is presumably the D108 *mod* transcript which requires the host *dam* methylase in order to be expressed. These results are consistent with those obtained by Hattman (21) and Plasterk *et al.* (11) who noted the same result for the Mu *mom* transcript. Sequence analysis of the D108 *mod* promoter region shows it to be nearly 100% homologous to the Mu *mom* promoter (34) (Fig. 1). The three *dam* methylation sites found in Mu also exist in D108, at the same locations with respect to the start of the *mod* gene. Furthermore, D108 probably also makes the 7 kDa protein (the *com* gene product), first postulated (10), and later shown by Kahmann *et al.* (35) as having a role in the control of *mom* expression, since the perfect Shine-Dalgarno sequence, as well as the initiator ATG codon, are conserved in D108.

We have also identified a 500 nt transcript which can be detected late in the lytic cycle in both *dam*⁺ and *dam*⁻ thermoinduced D108cts10 lysogens. There seems to be more of this transcript made in *dam*⁺ lysogens, possibly due to transcriptional activation of the 520 bp insertion promoter by the *mod* transcript. It is highly probable that this transcript originates from within the 520 bp insertion, based on the promoter consensus sequences (30) observed in that region, and the size of the insert-specific transcript seen in induced D108 lysogens. This promoter is apparently not under *dam* regulation, as the 500 nt transcript can be detected late in the lytic cycle of induced *dam*⁻ D108 lysogens. However, this promoter appears to be a poor one, based on its poor match to promoter consensus sequences (30), the low abundance of the 500 nt transcript seen in induced D108 *dam*⁻ lysogens, and the low levels of β -galactosidase produced from *lac* fusions to this promoter (data not shown).

There does not appear to be any termination consensus sequence (36) before the beginning of the 520 bp insertion, nor is any found within this insertion. A possible terminator structure is found 40-50 bp from the right end, at the same location as the putative Mu *mom* transcription terminator (11). It should be noted that in D108 the stem associated with this terminator is 1 bp shorter than Mu's terminator stem. This would suggest

that both the *mod* and ORF transcript share the same 3' end, though the proteins translated from them are in different reading frames.

The 520 bp additional DNA sequence was also shown to encode a 12 kDa polypeptide, in good agreement with the predicted molecular weight of 10.9 kDa. The function of this 12 kDa polypeptide is still, at this time, unclear. It does not appear to be required for the growth of phage Mu in laboratory strains of *E. coli*, since Mu functions without it. Southern (37) blot analysis using an insertion-specific probe revealed no homologous DNA sequences in Mu, P1 or the *E. coli* chromosome (not shown). Transcription of the D108-specific open reading frame can be detected late in the D108 lytic cycle of induced *dam*⁺ or *dam*⁻ lysogens. However, there could certainly be less of the 12 kDa protein expressed in induced *dam*⁻ D108 lysogens, as the protein may be translated from the *mod* transcript as well as its own transcript in *dam*⁺ *E. coli*. Preliminary results (not shown) of *lacZ* fusions to the D108-specific open reading frame promoter suggest that constitutive expression can occur, at low levels, independent of *mod* transcription or other D108 regulatory circuits. It is quite possible that the 12 kDa protein may encode some "accessory" or non-essential function not required for normal phage development. Bacteriophage Mu has several non-essential genes found in both the early region (*cim*, *kil*, *gam*, *sot*, *arm*) and the late region (*gin*, *mom*). The function of the 12 kDa protein may allow the phage to grow in a different host or environment, like the *ral* gene of bacteriophage λ , (38) or the *ipl* gene of phage T4 (39). Another possible function may be the alteration of the cell wall or membrane, such as antigenic conversion, as seen with the *Pseudomonas aeruginosa* PAO1 phage D3 (40). When we infected strains expressing the 12 kDa protein with various phages, the growth of D108 was inhibited in cells expressing this protein, while the growth of Mu was only slightly inhibited, and the growth of phage P1 was unaffected. This inhibition of D108 growth was only observed after D108 infection, and not D108 lytic growth after induction, since thermoinduced D108cts10 lysogens expressing the 12 kDa protein still lysed normally and released viable phage. Furthermore, preliminary results indicated that the 12 kDa protein may reside in the periplasmic space as well as the cytoplasm (results not shown). We have chosen to call this open reading frame *ges*, for "gained extra sequence".

It is possible that Mu may have evolved from D108 by losing certain functions that have affected the growth of Mu in one host but not another,

thus making Mu a more "specialized" phage. It is known that the host range of D108 is broader than that of Mu, and more closely resembles that of P1 (2). The results presented here show that the differences in Mu and D108 are not solely due to different gene products, but also additional DNA sequences and gene products. These differences have allowed us not only to characterize Mu and D108 transposition and its regulation, but also their behavior as phages (5,12,41). While the similarities between these phages may tell us more about their general properties, the distinct and subtle differences between them may indicate how these phages have evolved in nature.

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*To whom correspondence should be addressed

Present addresses: ¹Institute of Genetics, University of Glasgow, Church Street, Glasgow G11 5J6, UK and ²Department of Endocrinology, Montreal Children's Hospital, 2300 Tupper Street, Montreal, Quebec H3G 1A4, Canada

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