ELECTRONIC LETTER

Genetic refinement and physical mapping of a biparental complete hydatidiform mole locus on chromosome 19q13.4.

M D Hodges, H C Rees, M J Seckl, E S Newlands, R A Fisher

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Hydatidiform mole (HM) is an uncommon type of pregnancy, typically resulting from a diandric conception. HM may be defined as complete (CHM), having an androgenetic, diploid genome (AnCHM),[1 2] or partial, having a diandric, triploid genome (PHM).[3 4] A rare type of CHM is diploid but, unlike AnCHM, is biparental (BiCHM) in origin.[5 6] It has been shown that AnCHM and BiCHM are pathologically indistinguishable,[7] such that the pathology of BiCHM is also likely to involve the misexpression of imprinted genes.[8]

Genomic imprinting has recently been investigated in BiCHM tissue. In one case of recurrent BiCHM, five maternally set, imprinted genes were shown to have a paternal methylation pattern in the molar tissue, while *H19*, a paternally set, imprinted gene, had a normal pattern of methylation.[9] A series of BiCHM from several patients have also been examined for expression of the *CDKN1C* gene that is dependent on a maternally set imprint of the *KvDMR1* control centre.[10] The *CDKN1C* gene product, p57[KIP2], was not expressed in AnCHM tissue as would be predicted from their purely paternal genome. However, the p57[KIP2] protein was also absent from BiCHM samples despite the presence of a maternal genome, implying failure to set the maternal imprint for *CDKN1C (KvDMR1)*.[11] These two studies show that the BiCHM gene is associated with a loss of maternal imprints within the ovum, which is not limited to one locus, as is the case with other genes that regulate imprinting in *cis*. Instead, the BiCHM gene may act as a major regulator of imprinting and act in *trans* throughout the genome.

Recurrent BiCHM has been shown to be familial,[9 11–15] with a mode of inheritance consistent with an autosomal recessive trait. In two affected families,[12 13] a maximum multipoint lod score of 3.98 at D19S210 was identified for a minimum homozygous region of 15.2 cM.[14] This candidate region at 19q13.4 was also found to be homozygous in an Italian family enabling a refinement of 2.8 cM at the proximal end.[15] In affected sisters of a fourth family (CX01) homozygosity for 19q13.4 was again found to segregate with the disease, but genotyping of the family did not narrow the critical region further.[11]

In this study, we have investigated the BiCHM 19q13.4 locus by genotyping a fifth family (CX02) with two affected sisters and seven unrelated women who had two or more BiCHM but no known family history of molar pregnancies. We report the genetic refinement of a BiCHM locus to an approximately 1.1 Mb physical contig of 19q13.4 and establish a transcriptional map over this region, containing several BiCHM candidate transcripts.

PATIENTS AND METHODS
Patients
Patients with recurrent BiCHM including family CX01 were identified as previously described.[7 11] Family CX02 is of European origin (fig 1). The proband, II.2, has had a single pregnancy, which was confirmed as a CHM on histopathological review. Her sister, II.1 was reported to have had two HM, six miscarriages, and one stillbirth. Histopathological review of the first molar pregnancy showed irregular dentate villi with focal circumferential trophoblastic proliferation and was diagnosed as a probable PHM while in the second pregnancy, budding villi with mild stromal debris and circumferential trophoblast proliferation suggested a CHM. The status of their sister II.3 is unknown, having had no pregnancies. Fluorescent microsatellite genotyping of tissue from both HM in II.1 and the single CHM in II.2 showed all HM to be biparental in origin with no evidence of trisomy[7 8] (data not shown). Immunostaining showed the cytotrophoblast cell nuclei and villous mesenchyme from the second HM of II.1 and the CHM in II.2 to be negative for p57[KIP2], consistent with a diagnosis of recurrent BiCHM (fig 2). Extravillous trophoblast cells in these sections showed p57[KIP2] staining providing an internal positive control.[16] Tissue was unsuitable for immunostaining from the earlier HM in II.1 owing to the predominance of degenerate villi.

Seven women, with recurrent BiCHM, but with no known family history of HM were also included in this study. CXS01 has been described previously.[7] CXS02 and CXS03 are of Asian origin while the remaining four patients (CXS04-7) are of white origin. All HM in these patients were confirmed as CHM on histopathological review and, where tissue was available

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Key points

- The aim of this study was to refine the position of the gene responsible for recurrent biparental complete hydatidiform mole (BiCHM), a rare autosomal recessive trait associated with a failure to set maternal imprints within the ovum.

- Homozygosity mapping has previously positioned the BiCHM gene to a 12.4 cM region of chromosome 19q13.4 in four unrelated families. In this study we have identified two subjects and a fifth family (CX02) with two affected sisters, all of whom were homozygous over this region.

- Using recently available sequence for chromosome 19q13.4 and novel polymorphic markers for this sequence we performed haplotype analysis of the CX02 family.

- The minimal region of homozygosity was refined to approximately 1.1 Mb, spanning markers AC29215 (D19S418) and AAAT11138.

- A transcript map was made across the refined region and several potential candidates for the BiCHM gene identified.
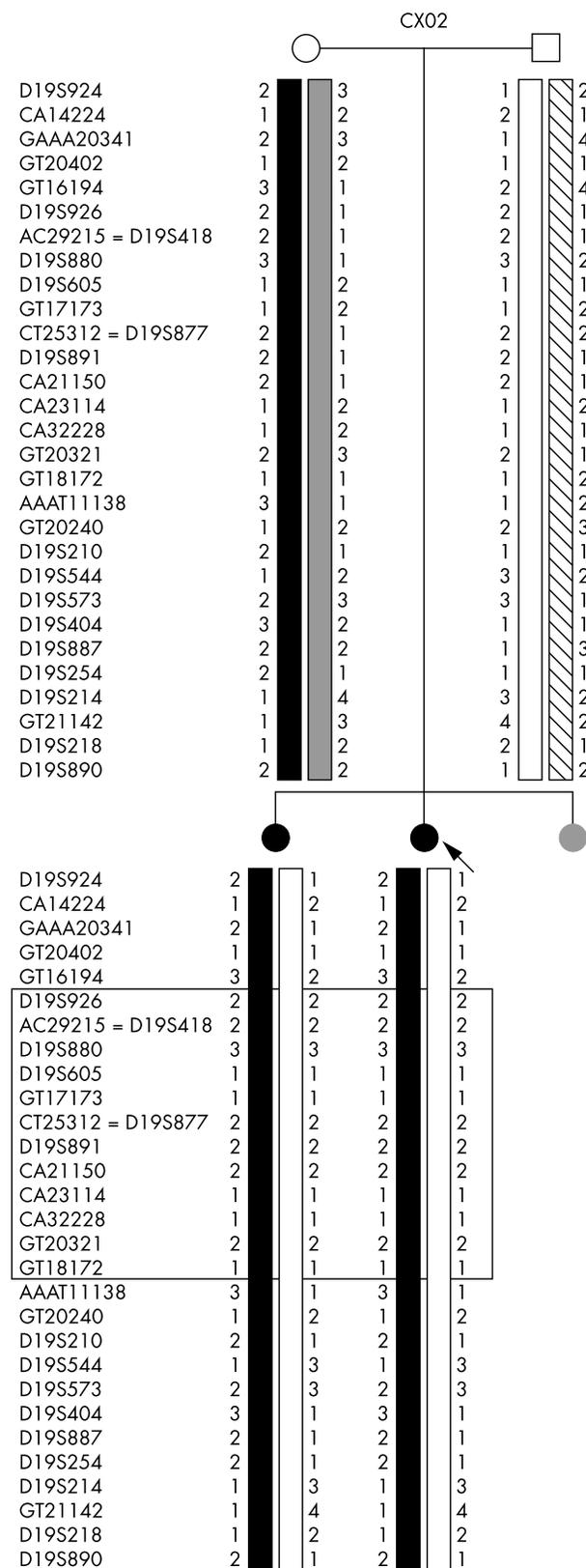
CX02

Marker				
D19S924	2	3	1	2
CA14224	1	2	2	1
GAAA20341	2	3	1	4
GT20402	1	2	1	1
GT16194	3	1	2	4
D19S926	2	1	2	1
AC29215 = D19S418	2	1	2	1
D19S880	3	1	3	2
D19S605	1	2	1	1
GT17173	1	2	1	2
CT25312 = D19S877	2	1	2	2
D19S891	2	1	2	1
CA21150	2	1	2	1
CA23114	1	2	1	2
CA32228	1	2	1	1
GT20321	2	3	2	1
GT18172	1	1	1	2
AAAT11138	3	1	1	2
GT20240	1	2	2	3
D19S210	2	1	1	1
D19S544	1	2	3	2
D19S573	2	3	3	1
D19S404	3	2	1	1
D19S887	2	2	1	3
D19S254	2	1	1	1
D19S214	1	4	3	2
GT21142	1	3	4	2
D19S218	1	2	2	1
D19S890	2	2	1	2

Marker				
D19S924	2	1	2	1
CA14224	1	2	1	2
GAAA20341	2	1	2	1
GT20402	1	1	1	1
GT16194	3	2	3	2
D19S926	2	2	2	2
AC29215 = D19S418	2	2	2	2
D19S880	3	3	3	3
D19S605	1	1	1	1
GT17173	1	1	1	1
CT25312 = D19S877	2	2	2	2
D19S891	2	2	2	2
CA21150	2	2	2	2
CA23114	1	1	1	1
CA32228	1	1	1	1
GT20321	2	2	2	2
GT18172	1	1	1	1
AAAT11138	3	1	3	1
GT20240	1	2	1	2
D19S210	2	1	2	1
D19S544	1	3	1	3
D19S573	2	3	2	3
D19S404	3	1	3	1
D19S887	2	1	2	1
D19S254	2	1	2	1
D19S214	1	3	1	3
GT21142	1	4	1	4
D19S218	1	2	1	2
D19S890	2	1	2	1

Figure 1 Haplotype analysis of family CX02 for 29 polymorphic microsatellite markers across chromosome 19q13.4. Haplotypes of parents (open symbols) and affected sisters (closed circles) are shown. A further sister (grey circle) has had no pregnancies and disease status remains unknown at this time. Marker AC29215 spans the polymorphic repeat defined by D19S418 and marker CT25312 spans the D19S877 repeat. The boxed region in the affected sisters represents an approximately 1 Mb region of homozygosity. Arrow indicates proband. Marker order was based on assembly of sequenced human chromosome 19 genomic clones.

for genotyping, shown to be biparental in origin. In all cases where immunostaining for p57[KIP2] was possible, the pattern of expression was the same as that seen in AnCHM (data not shown).

Genotyping and physical map
DNA was prepared from peripheral blood using standard techniques. PCR amplification and fluorescent microsatellite genotyping was carried out as previously described.[7] Markers selected spanned and included D19S924 to D19S890. Primer sequences and PCR amplification conditions were obtained from the Genome database (www.gdb.org). Thirteen additional primer pairs were subsequently designed to span polymorphic repeats based on the sequence of chromosome 19 genomic clones from within a contig of the 19q13.4 locus. Fluorescent PCR products were analysed using ABI 310 PRISM GeneScan software. Heterozygosity values for markers were taken directly from the Genome Database or, where data were not available, heterozygosity was determined by genotyping a control panel of 10 unrelated white and 10 unrelated Asian subjects. Heterozygosity values of novel markers were in the range 0.52 - 0.88 for both ethnic groups.

Computer analysis
Microsatellite marker sequences within the 19q13.4 region were used in BLAST searches against the non-redundant and high throughput genome survey, human genome sequence databases of the National Center for Biotechnology Information (NCBI). The chromosome 19 genomic clones identified were then assembled into a contig to span and include the entire BiCHM region from D19S924 to D19S890. Analysis of sequence within the contig was performed with the Nucleotide Identification X (NIX) programme, United Kingdom MRC Human Genome Project Resource Centre.[17]

RESULTS
Genetic refinement of a BiCHM locus
A physical map of the BiCHM 19q13.4 candidate locus was assembled from the NCBI databases.[18] [19] The parents and the two affected sisters from family CX02 were genotyped for 29 polymorphic repeats spanning this region (fig 1). This identified a region of homozygosity between markers D19S926 and GT18172 in both affected sisters that, based on the physical map of the region, was approximately 1 Mb (fig 3). Marker AAAT11138 was heterozygous and fully informative in all CX02 family members tested, refining the previous candidate region at the telomeric end by approximately 2 Mb. Marker GT16194 was heterozygous at the centromeric end in affected members from the family but did not further refine the centromeric boundary which has previously been defined by marker D19S418.[15] Affected sisters from family CX01 were homozygous for all additional markers tested (fig 3).

Seven women with recurrent BiCHM, but no known family history, were also genotyped. Two of these cases, (CXS01) and (CXS02), were found to be homozygous over the 19q13.4 BiCHM locus. CXS01 had a region of homozygosity between markers GT16194 and GT18172 and CXS02 a region spanning markers GAAA20341 and D19S218 (fig 3B). Four of the five subjects CXS03-07 were heterozygous for almost all markers in the refined region, with no single marker being uniformly homozygous in all cases (data not shown). A single patient CX04 was homozygous for three consecutive markers D19S210, D19S544, and D19S573.

Transcript map within the BiCHM candidate region
Analysis of the NCBI assembled sequence contig using the NIX program[17] identified approximately 60 gene loci in the candidate region. Seven transcripts from within this region (fig 4) were expressed predominately in germ cells, as
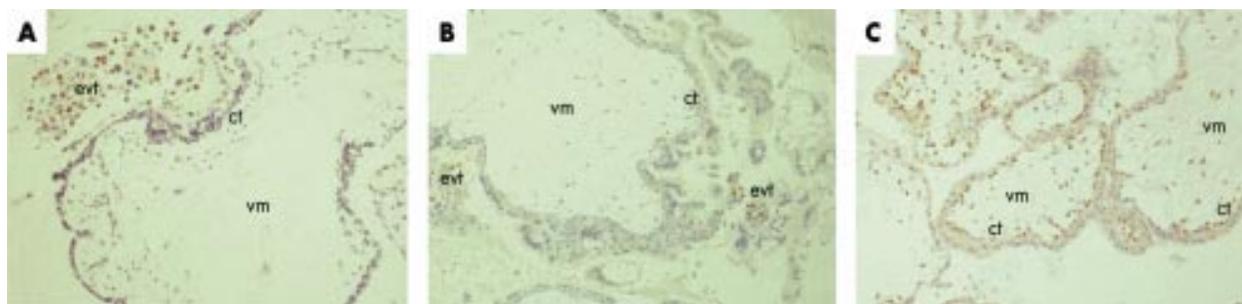
Figure 2 Immunostaining for p57[KIP2] in sections of tissue from BiCHM in affected members of family CX02 (A, B) and control tissue from a PHM (C). All sections have been counterstained with haematoxylin and eosin. (A). Tissue from the second HM from II.1 showing p57[KIP2] expression in extravillous trophoblast (evt) but not villous mesenchyme (vm) or cytotrophoblast cell (ct) nuclei. (B) Tissue from the BiCHM of the proband (II.2) with positive p57[KIP2] staining limited to the evt. (C) Tissue from a control PHM with positive p57[KIP2] staining in both ct nuclei and vm.
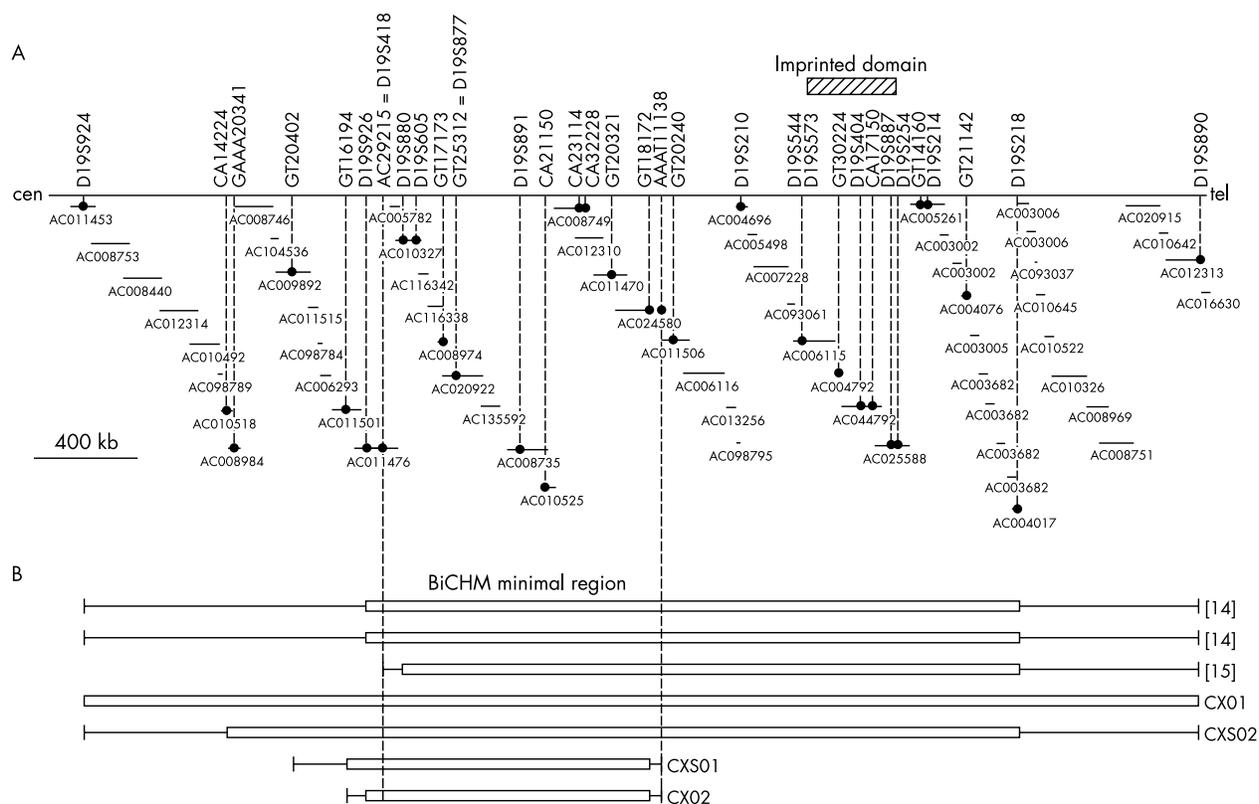
Figure 3 A 4.3 Mb genomic contig of chromosome 19q13.4 between markers D19S924 and D19S890, spanning the common regions of homozygosity in family CX02 and previously reported families with recurrent BiCHM.[11 14 15] (A) Contig of sequenced clones, with ordered microsatellite markers. Solid black circles indicate the specific clone within which the microsatellite marker is located. The physical length of each clone is indicated with a solid line and the accession number is indicated below. An imprinted domain, estimated to be approximately 400 kb, is represented by a hatched box. (B) Overlapping regions of homozygosity segregating with the disorder in families and subjects (CXS01, CXS02) with recurrent BiCHM. White bars represent regions where markers are homozygous. Lines indicate untested segments to adjoining heterozygous markers. Centromeric and telomeric boundaries of homozygosity were not determined for family CX01. A minimal region of approximately 1.1 Mb is common to all affected women homozygous for this locus.

determined by expressed sequence tagged (EST) analysis. These putative genes are therefore potential BiCHM candidates (table 1).

DISCUSSION

In this paper we report a further family (CX02) with the rare autosomal recessive condition recurrent BiCHM, a disorder mapped to a 12.4 cM region of 19q13.4 in four affected families.[11 14 15] As in the families that have been described previously, both affected sisters from CX02 had CHM of biparental origin. In addition, one sister had several miscarriages and a stillbirth. The family in the present study was not known to

be consanguineous. However, given the rarity of this condition, the affected subjects are likely to have inherited the mutation from a common ancestor and to be identical by descent over the region containing the mutated gene. Using a panel of previously described polymorphic markers for 19q13.4, we were able to confirm a region of homozygosity, segregating with the disease, in the two affected sisters. Combining these with a novel series of markers, we were able to saturate the 19q13.4 region and refine the maximum interval of homozygosity to approximately 1.1 Mb.

Seven further subjects who had two or more BiCHM, but without a family history of BiCHM, were also genotyped with the same markers. Two of them had regions of homozygosity,
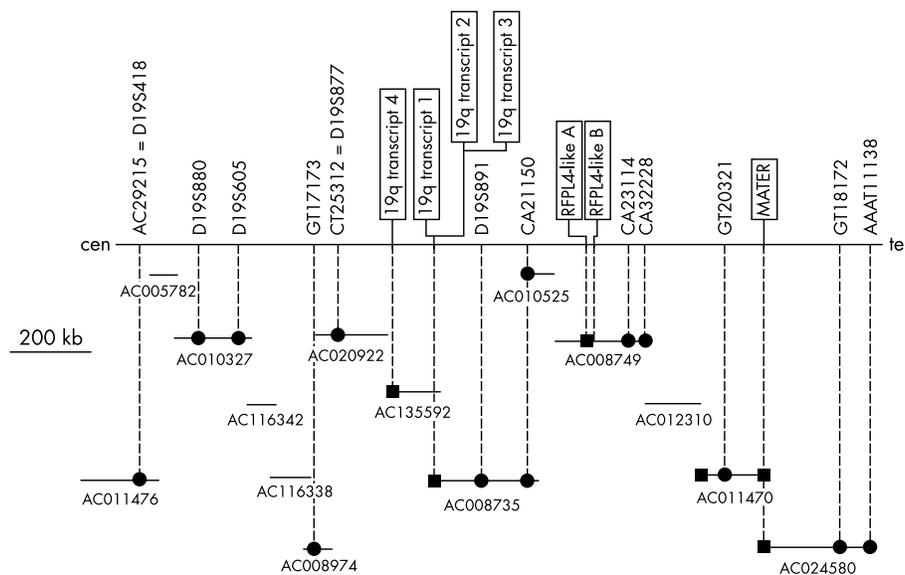
Figure 4 Refined genomic contig of the BiCHM critical region, spanning an approximately 1.1 Mb region from AC29215 to AAAT11138 on chromosome 19q13.4. Marker positions are indicated with black circles. Ovary, testis, or germ cell specific ESTs are boxed with dashed lines indicating the clone within which they map.

Table 1 Analysis of germ cell transcripts assigned to the BiCHM minimal region

Locus name	EST	Expression	BlastN	BlastP
19q transcript 1	AW590347 AI968795	Hs germ cell Mm oocyte, testis Rn neurone	Rn BF522719 (10^{-141}) Rn AI575717 (10^{-88})	Mm hypothetical XP_163149 (10-73)
19q transcript 2	BF514792 BF513988 BF194782 BF115988 BF941764	Hs ovary and pooled ESTs including ovary	None	No ORF detected
19q transcript 3	BG718783	Hs testis	None	No ORF detected
19q transcript 4	AW297892 BF061107	Hs ovary and pooled ESTs including ovary	None	No ORF detected
RFPL4-like A RFPL4-like B	None	Mm oocyte, testis	Hs XM_064986 (10^{-144}) Hs RFPL3 (10^{-24}) Mm RFPL4 (10^{-20})	Hs XM_064986 (10-110) Hs RFPL1 (10-94) Hs RFPL3 (10-92) Mm RFPL4 (10-84)
MATER (NALP5)	None	Mmu ovary Bt oocyte Mm oocyte	Mmu CB228956 (10^{-174}) Bt Z86039 (10^{-18}) Mm Mater (10^{-11})	Hs NALP4 (10-122) Hs NALP2 (10-106) Hs NALP1 (10-77)

Hs, *Homo sapiens*; Mm, *Mus musculus*; Rn, *Rattus norvegicus*; Bt, *Bos taurus*; Mmu, *Macca mulatta*.

of 1.2 Mb (CXS01) and 3 Mb (CXS02) respectively, overlapping that of the women with familial BiCHM[11 14 15] (this study). We believe that these subjects represent single affected members from families with recurrent BiCHM and that the gene defect in these women maps to the same 19q13.4 locus. However, genotyping of these two cases did not narrow the minimal region further. The telomeric boundary is therefore defined by marker AAAT11138 and the centromeric boundary by marker D19S418 (fig 3B). Five subjects with recurrent BiCHM (CXS03-07) were heterozygous across this region, although CXS04 was found to be homozygous for markers D19S210, D19S544, and D19S573. This region of approximately 250 kb was telomeric to the refined region but within that previously described as homozygous in other families with BiCHM. The parents of this subject were not available for genotyping. However, at two of these loci the alleles for which the person was homozygous were the most frequently occurring alleles, present at a frequency of 0.40 (D19S210) and 0.70 (D19S573) respectively in our white control panel.

The five subjects with recurrent BiCHM who lack homozygosity for the refined region may be compound heterozygotes for mutations in the BiCHM gene. Alternatively a second locus may be affected in these people. A previous report describing a woman with familial BiCHM,[9] who was also heterozygous across the 19q13.4 region, suggests that this condition may show genetic heterogeneity. Approximately 60 genes were localised to the 1.1 Mb refined interval (data not shown). It is known that maternal imprints are set within the ovum[20–23] and therefore BiCHM candidacy has been initially limited to putative genes that were primarily expressed in germ cells. Seven genes that met this criterion were identified (table 1). These include maternal antigen that embryos require (*MATER*),[24 25] a member of the NALP family of proteins thought to be associated with inflammatory pathways.[26] Fertilised ova of *Mater* null mice were found to arrest at the two cell stage of development, implying that the protein is essential for normal embryonic development.[27] We also identified two tandemly arranged putative genes with very similar sequence to each other and a

high degree of homology to the mouse ret-finger protein-like 4 (*Rfpl4*) gene. This E3 ubiquitin-protein ligase, which targets cyclin B1 for proteasomal degradation,[28 29] maps to the syntenic region of mouse chromosome 7. Based on this homology, these two genes have been initially termed *RFPL4-like A* and *RFPL4-like B*. A fourth candidate gene, 19q transcript 1, is defined by the two germ cell specific ESTs AW590347 and AI968795. This putative gene encodes an open reading frame (ORF) of 222 amino acids. Although it has a rat and mouse orthologue (table 1), there was no other recognisable database homology. Three further germ cell specific candidate loci were also identified (table 1). One of these is represented by a cluster of five ESTs that were found to span 1.1 kb of genomic sequence (19q transcript 2). No recognisable ORF was identified, raising the possibility that this cluster is within a non-coding region of a hitherto unknown transcript. The two other loci, also having no recognisable ORFs, were defined by single germ cell specific EST transcripts (table 1).

An imprinted domain has been mapped to chromosome 19q13.4[30 31] and it has been speculated that the defective gene in women with BiCHM might normally regulate expression of genes within this region.[14] This domain is approximately 400 kb, the most centromeric imprinted gene in this cluster being *ZIM2*, mapping to the chromosome 19 genomic sequence AC006115, and the most telomeric *ZNFP264*, mapping to AC025588 (fig 3A). The present study places this imprinted domain approximately 600 kb telomeric to the minimal BiCHM region (fig 3). Whether the BiCHM gene regulates expression of genes in this domain remains unproven. However, it has been clearly shown that the BiCHM gene does regulate expression of multiple imprinted genes on other chromosomes.[9 11]

To date, it is the *DNMT3L* gene[32] that fits many of the requirements of the BiCHM gene although *DNMT3L* is unlikely to be the BiCHM gene itself.[8 9 11] The murine Dnmt3L protein, essential for the establishment of maternal methylation imprints, has recently been shown to interact with both Dnmt3a and Dnmt3b de novo methyltransferases,[33] acting specifically through Dnmt3a to methylate DNA.[34] This complex was found to methylate DNA de novo irrespective of the target sequence and raises the possibility that a further factor, potentially the product of the BiCHM gene, is responsible for the correct targeting to maternally set imprinted genes. Alternatively the BiCHM gene could play a role in the Dnmt3L mediated recruitment of histone deacetylase 1 to maternally imprinted genes.[35 36]

Whether a transcript within the BiCHM minimal region either interacts with the methyltransferase complex described above or is involved in a novel pathway has yet to be elucidated. Ultimately the identification of genes involved in molecular pathways resulting in the development of BiCHM will help to provide new insights into maternal imprinting during early embryogenesis.

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Authors' affiliations
M D Hodges, M J Seckl, E S Newlands, R A Fisher, Department of Cancer Medicine, Imperial College London, UK
H C Rees, Department of Histopathology, Charing Cross Hospital, London, UK

Correspondence to: Dr R A Fisher, Department of Cancer Medicine, Faculty of Medicine, Imperial College London, Charing Cross Campus, Fulham Palace Road, London W6 8RF, UK; r.fisher@imperial.ac.uk

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