Insulator and silencer sequences in the imprinted region of human chromosome 11p15.5

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The imprinting of the genes on human chromosome 11p15.5 is thought to be controlled by two imprinting control regions located in two differentially methylated CpG islands upstream of the H19 gene (H19 DMR) and in intron 10 of the KCNQ1 gene (KvDMR). We have examined sequences in the human 11p15.5 genomic imprinted region for the presence of insulators and silencers using a position- and enhancer-dependent stable transfection assay. We have confirmed the existence of insulators in H19 DMR and discovered two novel insulators in the IGF2 gene. We have also found two novel silencer sequences; one is located in KvDMR, a region that is thought to contain the promoter for the KCNQ1OT1 transcript, and another is in the CDKN1C gene. We have demonstrated binding of CTCF protein in vitro to all the insulator and silencer sequences that we have detected. We discuss the differences in the regulation of imprinting controlled by the two imprinting control regions in chromosome 11p.

INTRODUCTION

While most mammalian autosomal genes are expressed from both chromosomes, a few are expressed from only one of the two alleles in a parent-of-origin-specific manner. Such genes are said to be imprinted (1–6). When an imprinted gene passes through the germline, it acquires a ‘mark’ that identifies the parental origin of that allele. A paternally imprinted gene is not transcribed when inherited from the father but is transcriptionally active when derived from the mother. Likewise a maternally imprinted gene is silenced when it is derived from the mother but is expressed when paternally inherited.

Imprinted genes tend to occur in clusters encompassing 1 Mb or more of DNA. Such clusters contain both paternally and maternally imprinted genes that are often intermingled with non-imprinted genes. The imprinting of a cluster of genes is thought to be regulated in cis by a sequence known as the imprinting control region (ICR). Two such clusters in the human genome have been extensively characterized. One cluster of mostly paternally expressed genes is located on chromosome 15. Deletions within this cluster cause Prader–Willi or Angelmann syndromes (7,8). The other cluster is located on the short arm of chromosome 11 (11p15.5, Fig. 1). Dysregulation of the imprinted genes in this region is associated with the congenital overgrowth disorder called Beckwith-Wiedemann syndrome (BWS) (9,10) and with a variety of malignancies.

BWS is associated with a variety of disturbances in imprinting of certain genes in the 11p cluster (11–14). About 20% of BWS patients have paternal uniparental disomy (complete or partial) for chromosome 11 (11p15–17). Up to 50% of BWS patients show biallelic expression of the IGF2 gene. The increased dosage of this fetal growth hormone may cause the fetal overgrowth that is characteristic of BWS. Another 50% of BWS patients exhibit dysregulation of imprinting at the differentially methylated KvDMR locus; they show loss of methylation of the maternal allele that is accompanied by loss of imprinting of the normally maternally imprinted KCNQ1OT1 transcript (11,18–20). A small proportion of patients (<5%) have point mutations in the CDKN1C gene (21–23) or translocations/inversions through the KCNQ1 (KvLQT1) gene (19,20).

The imprinting of the 11p region is thought to be controlled by two imprinting control regions: one (‘H19 DMR’) controls the imprinting of the telomeric IGF2 and H19 genes while the other (‘KvDMR’) is thought to control the expression of a centromeric cluster of genes that includes the KCNQ1 gene, its antisense transcript, KCNQ1OT1 (LIT1 or KvLQT1AS), and...
the cell cycle regulatory protein (p57) encoded by the CDKN1C gene (24, 25).

The understanding of the mechanisms of imprinting has been greatly enhanced by recent studies of two genes in Domain 1 of the 11p imprinted cluster (Fig. 1) and the syntenic region of mouse chromosome 7. In the human, the two telomeric genes in this cluster are H19 and IGF2. H19 encodes an apparently untranslated, maternally expressed RNA polymerase II transcript and IGF2 encodes insulin-like growth factor 2, a paternally expressed fetal growth factor. The promoters of these genes share a common pair of endodermal enhancers that are located 3' of the H19 gene (26, 27). They also share mesodermal enhancers that are downstream of the H19 gene (28). Furthermore, there is a differentially methylated region upstream of the H19 promoter (H19 DMR). Methylation of this DMR on the paternal allele and methylation of the adjacent H19 promoter block expression of the paternal copy by attracting the binding of methyl-binding proteins such as MECP2 and histone deacetylases like Sin3 (29–34). Moreover, the methylation of H19 DMR appears also to block the binding of the multivalent 11-zinc-finger protein, CTCF, to CTCF-binding sequences on the paternal chromosome (35–42). Absence of CTCF allows the paternal IGF2 promoter to utilize the downstream endodermal enhancers. The maternal copy of the DMR is unmethylated and hence is able to bind the CTCF protein. The CTCF-bound DMR acts as an enhancer-blocking sequence (insulator) and blocks the access of the IGF2 promoter to the downstream enhancers. Hence the maternal allele of IGF2 is not expressed. The mouse H19 DMR sequence has enhancer-blocking (insulator) activity when assayed in vectors designed to detect position-dependent gene expression. This insulator activity is detected when the sequence is between a promoter and its enhancer and correlates with the ability of the sequence to bind the CTCF protein in gel mobility shift assays in vitro.

Differentially methylated regions are a common feature of imprinted genes that may control imprinting by the differential binding of methylation-sensitive proteins such as CTCF and perhaps others such as BORIS (43) and CGBP (44). We therefore decided to search for other insulator sequences in 11p15.5 in order to uncover more information about regulation of the imprinted genes in this cluster. Here we report that, like the mouse sequences, the human CTCF-binding sequences upstream of H19 have enhancer-blocking (insulator) activity. We also found insulator activity located in two regions of the IGF2 gene: one in a CpG island that includes the promoter for a paternally expressed antisense IGF2 transcript called PEG8 and another in a highly conserved stem-loop structure in

Figure 1. The imprinted region of human chromosome 11p15.5. Genes and their imprinting status on human chromosome 11p15. The maternal (Mat) and paternal (Pat) chromosomes are shown. Grey boxes indicate maternally expressed genes and boxes filled with vertical lines indicate paternally expressed genes. The official names and some alternate gene names are shown. Some genes (e.g. TSSC4, TAB81, ASCL2, NAP1L4) are imprinted in the mouse but not the human. MTR1 is imprinted in human but not mouse. The direction of transcription is indicated for each gene by the arrows. The bars filled with diagonal lines indicate the locations of insulators or silencers as found in this work. Domains 1 and 2 are indicated by the horizontal arrows at the top of the figure. The dashed arrow indicates that domain 2 in the mouse extends further to the right. The circles to the right of H19 are the 3' enhancers used by H19 and IGF2. Some translocations through the KCNQ1 gene cause BWS. Tel = telomeric end of 11p.
intron 7. We also found strong silencer activity in several parts of the CDKN1C gene (a gene that is differentially methylated in the mouse though not in the human) and in KvDMR located in intron 10 of the KCNQ1 gene. This region is thought to contain the promoter for the KCNQ1OT1 transcript. This region's differential methylation and imprinting are disrupted in over half of the cases of BWS (11,18–20). We propose that imprinting in domain 1 is controlled by insulators whereas bidirectional silencers dominate imprinting in domain 2.

RESULTS

Insulators from human 11p: domain 1

Insulators have been defined by their ability to act as a barrier between a gene and the silencing effects of adjacent condensed chromatin or to insulate a promoter from the stimulatory effects of an adjacent enhancer (45). To assay several sequences for insulator activity, we used the test vector system developed in the Felsenfeld group (39,40). This vector ("pNL", Fig. 2A) contains the Neomycin-resistance (NeoR) gene driven by the human \(^\alpha\gamma\)-globin promoter and stimulated by the mouse HS2 enhancer of the \(\beta\)-globin LCR. Fragments to be tested (Fig. 2B) were placed between the promoter and enhancer ("enhancer-blocking or insulator site"), the DNAs were used to transfect human K562 cells and the numbers of G418-resistant colonies were measured. Insulator activity is indicated by a decrease in the number of G418-resistant colonies. We normalized the colony number to the number obtained with the pNI vector without any insertions. The pNIDMD vector (40) that contains the 1.6 kb mouse \(H19\) DMR (also called \(H19\) DMD) cloned into the insulator site showed an 8-fold reduction in the number of G418-resistant colonies (Fig. 2C), consistent with the results obtained by Bell and Felsenfeld (40). We define the strength of the insulator activity as the fold decrease in the number of colonies. The vector that lacked the enhancer gave a 21.5-fold reduction whereas a control 2.3 kb fragment from phage \(\lambda\) DNA and a 986 bp fragment from the Cpg island-containing promoter region of the maternally imprinted ZNF127 gene on chromosome 15 gave no insulator activity (data not shown). This shows that the insulator activity is not simply the result of changing the distance between the promoter and enhancer. These results essentially corroborate those from the Felsenfeld group.

\(H19\) DMR. The \(H19\) DMR (DMR) regions upstream of the mouse and human \(H19\) genes have been shown to have insulator activity. To confirm these results for the human DMR we assayed three fragments (Fig. 2C, labeled \(H19\)-A\(_i\), \(H19\)-B\(_i\), \(H19\)-C\(_i\)) covering the DMR upstream of the human \(H19\) gene for insulator activity; these include several repeats (41) that contain consensus sequences for the binding of CTCF (46). These sequences are conserved between the human and mouse. As can be seen in Figure 2C, the 1258 bp \(H19\)-C\(_i\) fragment gave strong orientation-dependent insulator activity (4.8-fold reduction in forward orientation), but none in the reverse orientation. This fragment contains the CTCF `B1'consensus (41) and the H7 consensus of Bell and Felsenfeld (40). The 958 bp \(H19\)-B\(_i\) fragment containing three B repeats, 1–3, showed weak insulator activity in either orientation (~2–3-fold reduction), whereas the 1089 bp \(H19\)-A\(_i\) fragment containing the three B5–7 repeats exhibited reproducible insulator activity in either orientation (Fig. 2C). This result is similar to that obtained previously (42).

To determine whether the \(H19\) fragments had silencer activity in addition to their insulator activity, we also cloned them into the Ndel site of the vector and repeated the assays. As can be seen in Figure 2D (top three bars), all these fragments displayed less than a 2-fold decrease in colony number, regardless of their orientation. This agrees with the results obtained for the mouse \(H19\) DMR (40) and for the human DMR (41) and indicates that they are behaving as insulators rather than silencers.

\(IGF2\) gene. Approximately 25–50% of sporadic cases of BWS display biallelic expression of the \(IGF2\) gene. About half of these patients have paternal uniparental disomy of chromosome 11 and very few have hypermethylation of the \(H19\) DMR (11). Therefore half of BWS patients showing biallelic expression of the \(IGF2\) gene have no known cause for this biallelic expression. In order to identify new regions with assayable phenotypes in these patients, we tested two regions of the human \(IGF2\) gene for insulator activity. The human \(IGF2\) gene contains a differentially maternally methylated region in a Cpg island that encompasses an antisense, paternally expressed transcript called PEG8 (Fig. 2B) (47–49). This Cpg island is near the P2-P4 promoters. The 1660 bp \(IGF2\)-B\(_i\) fragment derived from these sequences gave strong insulator activity (Fig. 2C) regardless of the orientation.

Intron 7 of the human \(IGF2\) gene contains a putative stem–loop structure that is conserved in human, mouse, rat and horse (Fig. 2B) (50). Although this region is located in an area that is differentially paternally methylated in the mouse (49,51), its methylation status in humans is unknown. This region (fragment IGF2-A\(_i\)) also contained strong insulator activity in both orientations (Fig. 2C). Neither of these \(IGF2\) fragments was active when placed in the silencer position of the vector (Fig. 2D), regardless of their orientation.

Silencers from human 11p: domain 2

\(KCNQ1\) gene. KvDMR is a differentially methylated region in intron 10 of the \(KCNQ1\) gene. This region is thought to be near the promoter of the \(KCNQ1OT1\) transcript, a maternally imprinted transcript that is in antisense orientation to the \(KCNQ1\) gene. This region is maternally methylated and paternally unmethylated. In over half of the cases of BWS there is loss of methylation of the maternal KvDMR accompanied by biallelic expression of the \(KCNQ1OT1\) transcript (Fig. 1) (11,18–20). We therefore assayed a 1486 bp KvDMR-A, fragment (Fig. 3A) containing KvDMR for insulator or silencer activity. The fragment in the forward orientation gave strong insulator activity (Fig. 3B). The reverse orientation also gave substantial activity. Subfragments of the 1486 bp fragment (Fig. 3A) also showed blocking activity when cloned into the AscI site of the vector (Fig. 3B). The strongest activity was shown by an 857 bp subfragment (KvDMR-E\(_i\)), whose activity was as potent as the entire fragment. The other subfragments...
showed lesser activities, some of which were orientation-dependent. The KvDMR-Ai fragment also had substantial orientation-independent silencer activity when placed into the NdeI site (Fig. 3C). That shown by the subfragments KvDMR-Bi and KvDMR-Ei was less than their insulator activity (Fig. 3C).

**CDKN1C** gene. The **CDKN1C** gene is contained in a large CpG island that is differentially methylated in mouse but not in human (52). However, this gene has been shown to acquire biallelic methylation in certain human tumors (53,54). Mutations in this gene are found in <5% of sporadic cases of BWS but account for 25–50% of familial cases (21–23,55). The second intron contains two regions that are strongly conserved between the mouse and human. Therefore we assayed several fragments from this gene for insulator activity. The 2.05 kb CDK-Ai fragment that includes exons 1 and 2 (Fig. 4A), gave a 6-fold reduction of colonies in either orientation (Fig. 4B). In an attempt to localize the insulator activity, we assayed several smaller fragments of the **CDKN1C** gene. Insulator activity was found for all six fragments from **CDKN1C** (Fig. 4B), although the activity was slightly less than that shown by the entire fragment. The activity was largely orientation-independent. It appears that several regions of the gene contribute to the insulator activity of the 2.05 kb fragment.

**Figure 2.** Insulator and silencer activity of the gene fragments from Domain 1 of human chromosome 11p15.5. (A) Vector used in the insulator/silencer assay (40). The plasmid contains the human &gamma;-globin promoter (horizontal arrow) driving the NeoR reporter gene (black rectangle) and stimulated by the mouse HS2 enhancer of the &beta;-globin LCR (gray oval). The test fragment is cloned into either the Aci (blocking or insulator) or NdeI (silencing) site and the plasmid, linearized at the SalI site, is used to transfect stably K562 erythroleukemia cells in the presence of G418. The construct also contains an insulator from the chicken &beta;-globin gene (white rectangle) to prevent position effects arising from tandem integrations of the vector. (B) Schematic map of gene fragments tested for enhancer-blocking activity. The partial gene structures and the locations of the tested fragments are shown. Open blocks in IGF2 represent exons while the gray blocks upstream of H19 are repeat sequences B1–B7, A1 and A2 (42). The filled blocks represent the CTCF-binding consensus sequences as defined in (42). The bracketed part of each gene indicates the extent of the fragments tested (see Table 1). Solid arrows indicate the direction of the sense transcription while the dashed arrow shows the antisense transcript. (C) Enhancer-blocking activity as measured by colony formation assay. Constructs with the test fragments in the blocking site (Aci site) of vector pNI were transfected in triplicate into K562 cells and colony numbers were determined 3 weeks after plating on soft agar. The numbers of G418-resistant colonies for each construct are normalized to non-insulated control vector pNI and the numerical value of blocking activity relative to the vector control (fold reduction). The fold reduction in colony number is given by the number adjacent to each bar. The forward and reverse orientations were as defined in the Methods. In Figures 2–4, the error bars are standard errors from at least three experiments. (D) Silencer activities of domain 1 fragments. The same fragments tested in (C) were cloned into the silencer (NdeI) site of the pNI vector and assayed for colony formation in K562 cells in the presence of G418. The normalized fold reduction in colony number is shown. Little silencer activity was seen.
the KCNQ1 intron 10 region. The entire fragment showed strong orientation-independent, silencer activity. Subfragment CDK-Bi showed strong silencer activity in the reverse orientation and weak activity in the forward orientation. Subfragment CDK-Ei (forward orientation) showed strong silencer activity, although we were unable to clone this fragment in its reverse orientation. The CDK-Gi fragment also showed weaker, but reproducible silencer activity.

In summary, fragments from the H19 and IGF2 genes in domain 1 showed insulator activity without accompanying silencer activity. Fragments from the CDKN1C and KCNQ1 genes in domain 2 showed silencer activities when placed either between the insulator and enhancer or downstream of the enhancer.
enhancer. Therefore silencer activity is attributed to these fragments.

**Binding of CTCF protein in vitro to DNA fragments containing insulator or silencer activity**

In order to provide evidence for a possible role of the CTCF protein in the insulator or silencer activity, we looked for the ability of the CTCF protein to bind in vitro to the fragments showing insulator or silencer activity in vivo. The CTCF protein is a multivalent 11-zinc-finger protein that has a rather degenerate recognition sequence (35,37,56–58). Thus it is difficult to recognize its binding sites merely by inspection of a given DNA sequence. We therefore synthesized the protein in vitro in a rabbit reticulocyte lysate, incubated the suitably programmed lysate with a [32P] end-labeled DNA fragment, and analyzed the DNA-protein complexes by polyacrylamide electrophoresis (Fig. 5, lanes 4–6, all panels). In all experiments, we ran a control in which the fragment was incubated with an unprogrammed reticulocyte lysate (Fig. 5, lanes 1–3, all panels). As a positive control we used the 274 bp DNA fragment containing the human B1 binding site from the H19 DMR (42) [Fig. 5, panel A(i)]. We also used a double-stranded oligonucleotide containing this sequence as a competitor to ensure that any binding was specific to the CTCF protein (Fig. 5, lanes 2, 5, all panels). As an additional control we used an oligonucleotide containing a mutated CTCF consensus as a competitor (Fig. 5, lanes 3, 6, all panels). As can be seen in Figure 5A, the programmed lysate caused a shifted electrophoretic band (frequently a doublet) that was not present with the unprogrammed lysate (lanes 1 and 4) and whose presence was inhibited by the addition of an excess of unlabeled oligonucleotide (lanes 2 and 5). The mutated oligonucleotide did not abolish the appearance of this band (lanes 3 and 6). An example of a fragment from CDKN1C (CDK-J_R) that showed non-specific binding by the unprogrammed lysate is shown in Figure 5A(ii). To further corroborate that the mobility shift was due to binding of the CTCF synthesized in vitro, we incubated the programmed or unprogrammed reticulocyte lysate with anti-CTCF antibodies or pre-immune γ-globulin before addition of the radiolabeled probe. The CTCF antibody but not the pre-immune γ-globulin supershifted or inhibited the appearance of the band caused by the programmed but not the unprogrammed lysate (data not shown). Bands caused by the unprogrammed lysate were not inhibited or supershifted.

We then proceeded to assay various subfragments from the regions that showed blocking or silencing activity in the in vivo assay. A 151 bp subfragment from the IGF2 stem loop region, fragment (IGF2-C_H), gave a CTCF-dependent, competent mobility shift [Fig. 5B(ii)], whereas the larger IGF2-A, fragment could not be assayed because it gave a non-specific mobility shift (data not shown). In the IGF2 CpG island, the 174 bp fragment (IGF2-D_R) showed a CTCF-dependent mobility shift that was competed by the wild-type but not the mutant B1 oligonucleotide [Fig. 5B(ii)].

Three fragments from the KCNQ1 region gave specific, competent, gel mobility shifts [fragments KvDMR-F_D-R, -G_D-R, -H_D-R. Fig. 5C(i)-(iii)]. These fragments overlapped those that gave insulator or silencer activities. Similarly, four PCR fragments from the CDKN1C gene gave specific gel mobility shifts [CDK-H_B, -I_B, -K_B, -L_B, Fig. 5D(i)-(iv)]. These fragments overlapped the subfragments that gave positive insulator and silencer activities (subfragments CDK-B_R, -E_R, -G_R).

In summary, all the fragments that registered insulator or silencer activity in the K562 erythroleukemia cell assay in vivo contained sequences that were able to bind the CTCF protein in vitro.

**DISCUSSION**

The data reported in this paper show that several sequences in the imprinted region of human chromosome 11p15.5 possess insulator or silencer activity when assayed in vivo in K562 human erythroleukemia cells. The assay has been used to detect insulators in the chicken β-globin gene and in the mouse DMR region upstream of the H19 gene. We show that the activity is not simply a distance effect caused by lengthening the distance between the promoter and the enhancer. Furthermore the effect does not occur when we place any CpG-rich sequence between the promoter and enhancer as such a region from the maternally imprinted ZNF127 gene has no such activity.

**Insulators in domain 1**

We have confirmed the results of Hark et al. (42) that the H19 DMR in the human has similar insulator activity. We also found by gel mobility shift assays that this region binds the 11-zinc-finger protein, CTCF. These results support the model first proposed in the mouse that the reciprocal imprinting of the IGF2 and H19 genes in domain 1 is mediated by binding of CTCF protein to the non-methylated maternal H19 DMR.

Since many patients with Beckwith–Wiedemann syndrome show biallelic expression of the IGF2 gene with no known cause, we searched for sequences with insulator activity in that gene. We found that two regions in IGF2 also have strong insulator activity. One is in a highly conserved palindromic sequence located in intron 7 of IGF2. Although the methylation status of this region in the human is not known, the homologous region in the mouse is thought to be differentially paternally methylated (49,51). Furthermore, the mouse sequence was shown to bind a factor from a crude nuclear extract from mouse neonatal liver in a methylation-sensitive manner. Therefore, it is possible that the human stem–loop structure could act as an insulator if the putatively non-methylated maternal allele bound to a methylation-sensitive DNA-binding protein such as CTCF. This mode of insulation would be analogous to that provided by the H19 DMR. Although we could not demonstrate specific binding of CTCF to the oligonucleotides containing the putative stem loop (50) (L.G.B., data not shown), we were able to find CTCF binding elsewhere in the 324 bp IGF2-A, fragment. Therefore, it would be informative to look for differential methylation in this region and for aberrations of methylation in BWS patients with biallelic expression of IGF2. A small proportion of BWS patients who show biallelic expression of IGF2 have hypermethylation of the maternal H19 DMR whereas most patients do not (11). It is possible that some BWS patients
Figure 5. Detection of CTCF binding activity by gel mobility shift assays. [$^{32}$P]end-labeled fragments were incubated with unprogrammed (lanes 1–3) or programmed reticulocyte lysate (lanes 4–6) and analyzed by PAGE as described in the Methods. Reactions contained no competitor (lanes 1 and 4), B1 oligonucleotide competitor (lanes 2 and 5) or mutated B1 competitor (lanes 3 and 6). The fragments and primers used to generate them are shown in Table 2. (A) (i) Fragment H19-B1PCR used as a positive control. (ii) An example of non-specific binding of fragment CDK-JBi is shown. (B) (i) Fragment IGF2-CBi. (ii) Fragment IGF2-DBi. The locations of the fragments used for binding experiments (‘Bi’, brackets) are shown in the schematic to the right along with the fragments used in the insulator assay (‘i’, double-headed arrows). (C) (i)–(iii) Binding of KvDMR fragments by CTCF along with schematic showing their relationship to the fragments used in the insulator assays. (D) (i)–(iv) Binding of CDKN1C fragments by CTCF accompanied by a schematic showing their relationship to the fragments used in the insulator assays (bottom).
would show methylation of the maternal allele of this conserved region in the IGF2 gene.

The second insulator sequence was in a maternally methylated ~4 kb CpG island that is near the P2–P4 promoters of the IGF2 gene and that also overlaps the promoter of the IGF2 antisense gene called PEG8 whose transcription initiates between human IGF2 exons 4 and 4’. This region is differentially methylated on the maternal allele (48). Since the region with insulator activity is downstream of P2, overlaps P3 and is upstream of P4, it is unlikely to insulate the P3 and P4 promoters from the 3’ enhancers. Instead, it may function to insulate the paternal P3 and P4 promoters, (the major promoters for imprinted IGF2 transcription) (47) from enhancers or silencers that lie upstream of them and might alter imprinted expression of IGF2. Alternately, the non-methylated paternal allele may bind to transcription factors like CTCF that stimulate imprinted transcription from the P3 and P4 promoters. The CTCF protein is known to act as both an inhibitor and an activator of transcription depending on the context of its binding sites (35,57–60).

The role of the PEG8 paternally expressed transcript in the imprinting of the IGF2 gene (49), if any, is unknown. However if it does have any role, then the insulator activity that we found may insulate the PEG8 promoter from putative enhancers or silencers that are telomeric to the PEG8 promoter.

Silencers in domain 2

We found sequences in KvDMR and in the CDKN1C gene that acted as silencers in the K562 cell assay. While the H19 DMR is thought to regulate the reciprocal imprinting of only two genes, KvDMR may regulate the imprinted expression of up to six maternally expressed genes in domain 2. Deletion of KvDMR in human paternal chromosome 11 or in a paternally inherited syntenic mouse distal chromosome 7 led to silencing of several imprinted genes in this region, including the KCNQ1 and CDKN1C genes (24,25). As well, the KCNQ1OT1 transcript was lost. KvDMR is differentially methylated in both human and mouse. Therefore, if it acts as an insulator like the H19 DMR, the paternal allele should be bound by CTCF (or other proteins), to insulate the maternal allele of genes from putative enhancers. The promoters of the convergently transcribed KCNQ1 and CDKN1C genes are outside the KvDMR but since the location of such enhancers for these genes is uncertain, it is difficult to speculate about the viability of an insulator model for KvDMR. It should be noted that the KvDMR region of the mouse has been found to have insulator rather than silencer activity using an assay similar to the one we have used (61). The differences may reflect differences between the mouse and the human KvDMR sequences used. More recently, two reports appeared showing that the same region also possesses bidirectional silencing activity (62,63). Interestingly, the sequence triggered the spreading of methylation into adjacent sequences and the authors speculate that it is the result of the silencing process (63). Since the KvDMR region may include or be close to the promoter for the KCNQ1OT1 transcript, its regulatory role may be related to the transcript itself or the act of transcription of the KCNQ1OT1 region. Interestingly, the silencer activity found by Mancini–DiNardo et al. (62) did not appear to be simply due to the promoter activity.

The CDKN1C gene is in a CpG island that is differentially methylated in the mouse although not in the human (52). Because of the apparent absence of differential methylation in the human gene and because of the fact that both the CDKN1C and KvDMR sequences had silencer activity, the insulator model may not apply to the regulation of domain 2. It is possible that the imprinting control region in domain 1 functions via enhancer-blocking activity of one or more insulators but that imprinted expression of a number of genes in a domain as large as domain 2 relies on a series of bidirectional silencers such as those we have detected in CDKN1C and in KvDMR (Fig. 6).

In summary, we have discovered two regions in the human IGF2 gene that behave as insulators and two silencers that are located in KvDMR and the CDKN1C gene when assayed in tissue culture cells using artificial reporter constructs. Elucidation of the roles of these sequences in genomic imprinting in situ will require experiments with genetically engineered mice (25,64,65). We are investigating the roles of these sequences in imprinting of genes in domains 1 and 2 and their possible involvement in the pathogenesis of Beckwith–Wiedemann syndrome.

MATERIALS AND METHODS

Plasmid constructs

The plasmids pNI and pNIDMD were a gift from Dr Gary Felsenfeld (40). The former contains the human β2-globin promoter driving the NeoR reporter gene and stimulated by the mouse HS2 enhancer of the β-globin LCR, whereas the latter contains, in the AscI site between the promoter and enhancer, the mouse H19 differentially methylated region that is known to possess strong enhancer-blocking activity (Fig. 2). The pNIAE in which the enhancer has been deleted by EcoRI digestion and religation was the gift of Dr Marc Shulman and Peter Azmi.

The PCR primers used to amplify the fragments used in this work are shown in Tables 1 and 2. The fragments are named according to their gene of origin (H19, IGF2, KvDMR, CDKN1C) as well as their use: ‘i’ = insulator assay, ‘u’ = CTCF-binding assay. Most of the gene fragments to be tested [except for the 2046 bp CDKN1C fragment (CDK-Ai, see below)] were first PCR-amplified using the primers listed in Table 1 and the PAC DNA template that contained the human H19 gene (PAC DJ0988N23), the human IGF2 gene (PAC DJ0416J11) and the human KCNQ1 and KvDMR sequences (PAC DJ0074K15). The fragment from the CpG island upstream of the human ZNF127 gene was amplified from human genomic DNA. The PCR reaction for the amplification of the H19 fragments, the IGF2 stem-loop structure, the IGF2 CpG island, the KvDMR fragment, and the ZNF127 CpG island-containing fragment contained: 1× PCRx amplification buffer, 1× PCRx enhancer solution (both from Invitrogen), 3.0 mM MgSO₄, 0.2 mM dNTPs, 4 μM primers, 20 ng template PAC or genomic DNA, 2.5 U of Taq polymerase (Invitrogen) in a volume of 100 μl. For all DNAs except the IGF2 CpG island
DNA, the amplification protocol was as follows: denaturation at 95°C, 2 min, followed by 30 cycles at 95°C for 45 s, 60°C for 30 s, 72°C for 2 min, and final elongation at 72°C for 10 min. For the IGF2 CpG island (IGF2-Bi) DNA, the ‘touchdown’ cycling protocol was 95°C for 4 min, 95°C for 30 s, 65°C for 30 s, 72°C for 1 min, repeat steps 2–4, 19 times reducing temperature of step 3 by 0.5°C per cycle, then 95°C for 30 s, 55°C for 30 s, 72°C for 1 min, repeat steps 5–7 14 times, final extension 72°C for 5 min. These amplified fragments were separated on an agarose gel, isolated using the Qiagen gel purification procedure and then cloned into the pCRII®-TOPO® vector (Invitrogen). To facilitate the subcloning of the fragments into either the enhancer-blocking site (AscI site) or the silencing site (Ndel site) of the pNI vector, those inserts that had been inserted into pCRII®-TOPO® were amplified with primers pr1359 and pr1360 (Table 1) that contained both the AscI and Ndel sites and whose 3` termini were complementary to sequences surrounding the multicloning sites in the vector. The 100 μl PCR reactions for all these fragments contained 1× PCRx amplification buffer and 12.5 μl of 10× PCRx enhancer solution (Invitrogen), 1.5 mM MgSO4, 0.2 mM dNTPs, 1.0 μM primers, 20 ng template DNA, 2.5 U Taq polymerase. The amplification protocol was 95°C for 3 min, 30 cycles at 95°C for 45 s, 60°C for 45 s, 72°C for 1.5 min, and final elongation at 72°C for 10 min. All these fragments were then digested with the appropriate restriction enzyme and were ligated to the AscI site or Ndel site of the pNI vector.

The 2046 bp CDKNIC gene fragment (CDK-Ai) was amplified from PAC DJ0754H15 with primers pr1339 and pr1340. The subfragments of KvDMR were generated by PCR with the following primers: KvDMR-Bi, pr1359 and pr1334; KvDMR-Ci, pr1335 and pr1336; KvDMR-Di, pr1337 and pr1338; KvDMR-Ei, pr1335 and pr1420. The CDKNIC gene subfragments were generated with the following primers: CDK-Bi, pr1339 and pr1341; CDK-Ci, pr1339 and pr1342; CDK-Di, pr1341 and pr1343; CDK-Ei, pr1340 and pr1344; CDK-Fi, pr1344 and pr1345. These were amplified from CDMKIC-containing plasmids.

Owing to the presence of an AscI site within KvDMR and CDKNIC intron 2 fragments, these fragments were liberated from the pCRII®-TOPO® vector by EcoRI digestion, the ends were filled with the Klenow DNA polymerase and the fragments were ligated to blunt-ended AscI-cut or Ndel-cut pNI vector. Similarly the 2.3 kb phage λ DNA HindIII fragment was cloned into the AscI site of the pNI vector. In addition, the KvDMR-Bi and -Ei, CDK-Bi, -Ei and CDK-Gi fragments were ligated to the Ndel-cut pNI vector.

The accuracy of the cloning as well as the orientation of the inserts were confirmed by DNA sequencing. The forward or reverse orientation of the fragment was arbitrarily defined in relation to the direction of transcription of the NeoR gene in the vector: the forward orientation of a fragment has the same transcriptional orientation in the genome as the NeoR gene in the vector and the reverse orientation has the opposite orientation.

Plasmid pBl1-1 was made by ligating gel-purified, annealed oligonucleotides pr1303 and pr1304 (Table 2) into pCRII®-TOPO® vector (Invitrogen) that had been cut with BamHI and EcoRI. This plasmid was used with M13F/R primers to amplify the 274 bp fragment H19-B1PCR, the positive control for the

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**Figure 6.** Model for imprinting control in domains 1 and 2 of the human 11p15.5 region. Top—maternal chromosome; bottom—paternal chromosome. Domain 1 is regulated by one or more insulators such as H19 DMR and those detected in this work in the IGF2 gene. Domain 2 is regulated by a series of bidirectional silencers (arrows) such as those in KvDMR and the CDKNIC gene. CH3 indicates methylation of the paternal H19 DMR (bottom) and the maternal KvDMR (top). The location of the enhancers for CDKNIC and KCNQ1 genes is unknown.
CTCF binding assays. The insert for plasmid p4E was amplified with primers pr213 (atc tcc gtg gac ccc gcg ac) and pr985 (act tct cag gcg ctg atc tc) from the CDKN1C-containing PAC DJ0754H15 (Table 2), using PCR reaction A and PCR program 4 except that the denaturation step was at 94°C for 1 min each cycle (see below). This plasmid was used as the template for fragment CDK-LBi in the mobility shift assays of the CDKN1C gene as well as for cloning the CDK-Gi fragment in the insulator/silencer assay.

Insulator/silencer assay

Human K562 erythroleukemia cells were obtained from the American Type Culture Collection. Assays were performed as described in (39,40). Briefly, all plasmid DNAs, prepared using Qiagen Midi Purification Kit, were linearized with SalI, phenol–chloroform extracted and ethanol-precipitated. The DNA concentrations were determined by both UV spectroscopy and analysis on an agarose gel. One hundred ng of genomic DNA of CDKN1C was used to program a rabbit reticulocyte lysate system (Ambion, IVT, catalog no. 1200) according to the manufacturer’s protocols with the exception that the translation mix was supplemented with 0.1 mM ZnSO4 (final concentration). An identical translation reaction without CTCF mRNA was used as a negative (unprogrammed) control. Four microliters of lysate were diluted 4-fold with 12 µl of a 1.33x concentrated buffer mixture that brought the final concentrations of components to 20 mM HEPES buffer (pH 7.5), 100 mM KCl, 2.5 mM MgCl2, 5% glycerol, 3 µg/ml single-strand herring sperm DNA, 100 µg/ml poly dI–dC, 1 mM DTT and 0.16 pmol of S-32P-labeled probe in a final volume of 16 µl. When used, unlabeled competitor oligonucleotides were present in 350-fold molar excess. The reactions were incubated at room temperature for 1 h before electrophoresis on a 4% polyacrylamide gel.

In vitro DNA binding by CTCF protein

The plasmid containing the full-length mouse CTCF cDNA was described by Hark et al. (42). The CTCF gene was excised using NcoI and XhoI and transferred into plasmid pCITE-4a(+) (Novagen) that had been cut with the same enzymes. The XhoI-linearized CTCF construct was used to synthesize capped messenger RNA with the Ambion Message Machine (no. 1344) that uses T7 RNA polymerase. After LiCl precipitation, the mRNA was used to program a rabbit reticulocyte lysate system (Ambion, IVT, catalog no. 1200) according to the manufacturer’s protocols with the exception that the translation mix was supplemented with 0.1 mM ZnSO4 (final concentration). An identical translation reaction without CTCF mRNA was used as a negative (unprogrammed) control. Four microliters of lysate were diluted 4-fold with 12 µl of a 1.33x concentrated buffer mixture that brought the final concentrations of components to 20 mM HEPES buffer (pH 7.5), 100 mM KCl, 2.5 mM MgCl2, 5% glycerol, 3 µg/ml single-strand herring sperm DNA, 100 µg/ml poly dI–dC, 1 mM DTT and 0.16 pmol of S-32P-labeled probe in a final volume of 16 µl. When used, unlabeled competitor oligonucleotides were present in 350-fold molar excess. The reactions were incubated at room temperature for 1 h before electrophoresis on a 4% polyacrylamide gel.
The fragments used in mobility shift assays were amplified by PCR using the primers listed in Table 2. The 50 µl reactions contained the following common constituents: 1x PCRx amplification buffer (Invitrogen), 0.25 mM dNTPs, 2 µM primers and 2.5 U of polymerase (Invitrogen). In addition, reactions A contained 2 mM MgSO4 and 2 TBE (42). The preincubation step used by these protocols all included an initial denaturation at 95°C for 3 min and a final extension at 72°C for 10 min. Cycling conditions were: (1) 35 cycles of 94°C for 1 min, 55°C for 45 s, 72°C for 1 min; (2) 35 cycles of 94°C for 1 min, 58°C for 45 s, 72°C for 1 min; (3) 30 cycles of 94°C for 45 s, 60°C for 45 s, 72°C for 90 s; (4) 35 cycles of 94°C for 45 s, 60°C for 45 s, 72°C for 1 min.

The following PCR reactions, amplification protocols and templates were used for each fragment: IGF2-CB1 and -DB1—A, 1 on PAC pDJ0416J11; CDK-HBi—B, 2 on the same clone as above; CDK-LBi—A, 3 on plasmid p4E (see above); KvDMR-FBi and -HBi—C, 4 on a previously-constructed plasmid containing a fragment of KCNQ1 intron 10; KvDMR-GBi—D, 4 on the same KCNQ1 plasmid.

The fragments used in the mobility shift assays for CTCF binding were chosen to contain the closest match to the 11 bp CTCF binding consensus as defined by Bell and Felsenfeld and by Hark et al. (40, 42). All fragments contained at least a 7/11 bp match to the consensus and one or more sequences of CCGN6GG. However, because of the multivalent mode of DNA recognition by the 11 zinc fingers of CTCF, its recognition sequence is highly degenerate (58).

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