*Proc. Natl. Acad. Sci. USA*
Vol. 92, pp. 12456–12460, December 1995
Medical Sciences

# Multiple genetic loci within 11p15 defined by Beckwith–Wiedemann syndrome rearrangement breakpoints and subchromosomal transferable fragments

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*Communicated by Bert Vogelstein, The Johns Hopkins Oncology Center, Baltimore, MD, August 14, 1995*

**ABSTRACT** **Beckwith–Wiedemann syndrome (BWS) involves fetal overgrowth and predisposition to a wide variety of embryonal tumors of childhood. We have previously found that BWS is genetically linked to 11p15 and that this same band shows loss of heterozygosity in the types of tumors to which children with BWS are susceptible. However, 11p15 contains >20 megabases, and therefore, the BWS and tumor suppressor genes could be distinct. To determine the precise physical relationship between these loci, we isolated yeast artificial chromosomes, and cosmid libraries from them, within the region of loss of heterozygosity in embryonal tumors. Five germ-line balanced chromosomal rearrangement breakpoint sites from BWS patients, as well as a balanced chromosomal translocation breakpoint from a rhabdoid tumor, were isolated within a 295- to 320-kb cluster defined by a complete cosmid contig crossing these breakpoints. This breakpoint cluster terminated approximately 100 kb centromeric to the imprinted gene *IGF2* and 100 kb telomeric to $p57^{KIP2}$, an inhibitor of cyclin-dependent kinases, and was located within subchromosomal transferable fragments that suppressed the growth of embryonal tumor cells in genetic complementation experiments. We have identified 11 transcribed sequences in this BWS/tumor suppressor coincident region, one of which corresponded to $p57^{KIP2}$. However, three additional BWS breakpoints were >4 megabases centromeric to the other five breakpoints and were excluded from the tumor suppressor region defined by subchromosomal transferable fragments. Thus, multiple genetic loci define BWS and tumor suppression on 11p15.**

Three lines of investigation point to a role for 11p15 in human cancer. (*i*) Beckwith–Wiedemann syndrome (BWS), which involves prenatal organ overgrowth and predisposition to several embryonal tumors, including rhabdomyosarcoma and Wilms tumor, maps to 11p15 by genetic linkage analysis (1, 2). (*ii*) 11p15 shows loss of heterozygosity (LOH) in the same group of tumors to which BWS patients are susceptible, as well as many adult tumors (for review, see ref. 3). We have demonstrated directly by genetic complementation the existence of a tumor suppressor gene within this band (4). (*iii*) At least two genes on 11p15, insulin-like growth factor II (*IGF2*; refs. 5 and 6) and the closely linked *H19* (5, 7), are imprinted, i.e., show parental origin-specific gene expression in normal development. Furthermore, *IGF2* shows loss of imprinting in embryonal tumors (5, 6, 8, 9).

The simplest hypothesis is that a single gene accounts for BWS and embryonal tumors and that balanced germ-line chromosomal rearrangements from BWS patients interrupt and, therefore, define this gene. Sait *et al.* (10) indirectly mapped by pulse field gel electrophoresis (PFGE) three such BWS breakpoints to a 675-kb region of 11p15 and >275 kb centromeric to *IGF2*. However, this distance is tentative as it was derived from the sum of several PFGE fragments, one of which was inferred from other larger overlapping PFGE fragments. Furthermore, two BWS breakpoints lie at an undetermined distance centromeric to these PFGE fragments (11). In these mapping studies, only one breakpoint has been isolated (10), and thus the precise physical relationship among them is unknown. Furthermore, indirect mapping by PFGE is limited by the large size of the fragments. Finally, the relationship between any of these breakpoints and a tumor suppressor gene on 11p15 has not been determined.

We cloned the region of 11p15 harboring eight BWS balanced germ-line chromosomal breakpoints to determine their precise physical relationship, to localize tumor-suppressing subchromosomal transferable fragments, and to determine whether the BWS and tumor suppressor loci coincide.

## MATERIALS AND METHODS

**Cell Lines.** The following cell lines from BWS patients with balanced germ-line chromosomal rearrangements were used: B10.1, with t(4;11)(p15.2;p15.4) (12); 1632, with t(9;11)(p11.2;p15.5) (13); B901, with t(11;22)(p15.5;q12) (14); B23.1, with t(11;12)(p15.5;q13.1); 1217, with t(11;16)(p15.5;q12) (15); CD2, with t(10;11)(p13;p15.5); WH5.3, with inv(11)(p15.4;q22.3) (12); and CV581, with inv(11)(p11.2;p15.5) (16). TM87-16 is a rhabdoid tumor cell line with t(11;22)(p15.5;q11.23) (17). Cells were cultured in RPMI 1640 medium and 10% (vol/vol) fetal bovine serum in 10% $CO_2$/90% air, with the exception of 1632 and TM87-16, which were cultured in Dulbecco's modified

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Abbreviations: BWS, Beckwith–Wiedemann syndrome; STF, subchromosomal transferable fragment; Mb, megabase(s); YAC, yeast artificial chromosome; PFGE, pulse-field gel electrophoresis; STS, sequence tagged site; FISH, fluorescence *in situ* hybridization; LOH, loss of heterozygosity.

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Eagle's medium with 10% fetal bovine serum in 5% $CO_2$/95% air.

**Sequence Tagged Sites (STSs) and Yeast Artificial Chromosome (YAC) Isolation.** STSs used to screen the Washington University human YAC library (18) were derived from the following probes within the region of LOH (3) in tumors and documented in the Genome Data Base (Johns Hopkins University): cCl11-10 (*D11S431*), cCl11-280 (*D11S466*), cCl11-289 (*D11S470*), cCl11-421 (*D11S657*), cCl11-440 (*D11S572*), cCl11-583 (*D11S738*), cCl11-598 (*D11S742*), cCl11-385 (*D11S551*), cCl11-565 (*D11S601*), cCl11-395 (*D11S648*), cCl11-469 (*D11S679*), cCl11-555 (*D11S724*), cCl11p15-19, pADJ762 (*D11S12*), H19S1 (*D11S813E*), pIGF2/8-1, phins310, cosINS/IGF2, L29 (*D11S501*), L163 (*D11S517*), ZnFP83 (*D11S776*), and ZnFP104 (11).

PCR primers derived by subcloning and sequencing after *Sau*3AI digestion were sequenced, except for *IGF2, H19*, and *D11S776*, which were derived from available sequences (respectively, GenBank accession numbers X03423 and M32053 and P.L., unpublished results). D112Y, B74Y, B115L, D122R, and B40L primers were constructed from YAC end clone sequences. PCR was performed as described (19). STSs were used for YAC screening as described (20). YAC PCR end clones were isolated by using bubble priming or ligation-mediated PCR as described (19).

**Fluorescence *in Situ* Hybridization (FISH).** High-resolution prometaphase chromosomes from peripheral lymphocytes of Epstein–Barr virus-transformed lymphoblastoid cell lines were obtained as described (13). The centromere-specific probe pLC11A (D11Z1) was used for identification of chromosome 11. Probes were labeled with biotin or digoxigenin by nick-translation. FISH with cosmids, YACs, and centromere-specific probes to chromosomes (21) and hybridization to extended DNA (22) were performed. Slides were examined under a Zeiss Axioplan epifluorescence microscope. A Cytovision Probe system (Applied Imaging) was used for digital imaging microscopy.

**Hybridization and Library Construction.** YAC-derived cosmid libraries were constructed as described (23). Probes for Southern and Northern blot hybridization were excised from gels and labeled by random priming (24). Hybridization and washes were as described (25).

**Subchromosomal Transferable Fragments (STFs).** Ninety-five STFs were constructed and analyzed as described (4). Each STF was initially hybridized with a panel of 24 probes throughout chromosome 11, and those STFs that contained 11p15 sequences were hybridized with an additional 17 probes from 11p15, to define their relative position with regard to BWS breakpoints. Rhabdomyosarcoma cell line RD-suppressing STFs (4) were hybridized with cosmids generated in this study to define their continuity and ends precisely.

**Identification of Transcribed Sequences.** Conserved fragments from cosmids and phage were identified by hybridization to Southern blots derived from dog, mouse, sheep, cow, rabbit, pig, chicken, fish, and frog DNA. Cosmids were also screened for *Bss*HII and *Not* I sites to identify potential CpG islands. Fragments identified by either method were hybridized to Northern blots prepared from a wide variety of adult and fetal tissues. In addition, YACs were used directly to screen a fetal kidney cDNA library prepared in λZap II, by using as a positive control the appropriate cosmid fragment recloned in λZap II.

## RESULTS

**Isolation of STSs and YACs Near BWS Breakpoints.** STSs were generated from 11p15 probes localized within the region of LOH in tumors. Single-copy subfragments from these probes were subcloned and sequenced to derive STSs for PCR-based screening of the Washington University human YAC library. These efforts led to the development of 19 STSs (deposited in the Genome Data Base). Thirty-six yeast strains were isolated by using these STSs. Twenty-four strains contained single nonchimeric YACs, five contained single chimeric YACs, and seven contained multiple YACs. The characteristics of 24 nonchimeric single YACs isolated from 11p15 are also deposited in the Genome Data Base.

**Identification of BWS Breakpoint Sites Within YACs.** FISH was used to determine whether any of the YACs crossed germ-line chromosomal rearrangements from BWS patients. YACs spanning seven breakpoints were identified in this manner. For example, the 11p15 breakpoint in BWS cell line WH5.3, with a chromosome 11 inversion, was spanned by YAC A39D9, as hybridization signals from the YAC were visible at both breakpoint sites of the inversion (Fig. 1*A*). Similarly, the breakpoint in BWS cell line B23.1, with a balanced (11;12) translocation, was spanned by YAC D112D9, as hybridization signals from the YAC were visible on both the derivative chromosome 11 and the derivative chromosome 12 (Fig. 1*B*).

Five BWS breakpoints were found to be clustered near but centromeric to *IGF2* and *H19* (Fig. 2*A*). As YAC probes were determined to be telomeric or centromeric or to encompass a breakpoint, the relative order of these five breakpoints was determined. In addition, the YACs themselves established precise upper limits on the distances between them. For example, as YAC D122D10 spanned the breakpoints in both B901, spanning a balanced (11;22) translocation, and B23.1 (Fig. 1B), these two breakpoints were separated by no more than 270 kb, the size of this YAC. Hybridization with known probes and with end clones derived from YACs by PCR amplification indicated that five YACs spanning these five breakpoints, and excluding *IGF2* and *H19*, formed a 700-kb overlapping YAC contig, representing a maximum distance among them at this level of resolution (Fig. 2*A*).

However, FISH analysis using 16 additional YACs established a minimum physical distance of 4.0–5.2 megabases (Mb) between the most centromeric of the cluster of five breakpoints

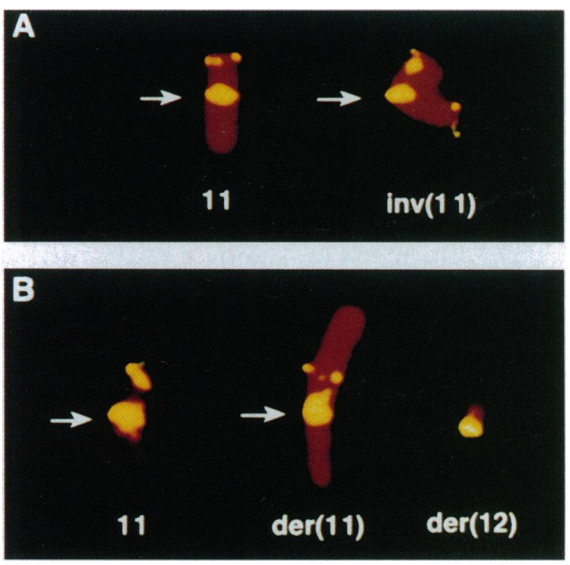


FIG. 1. Identification of YACs spanning BWS breakpoint sites. FISH was performed with YACs labeled by nick-translation on metaphase chromosomes from BWS patient cell lines. Arrows indicate a chromosome 11-specific centromere probe (26). (*A*) YAC A39D9 hybridized to BWS cell line WH5.3, with a chromosome 11 inversion. Signals are visible on the normal chromosome 11 as well as at both ends of the inversion, indicating that the YAC spans the breakpoint. (*B*) YAC D112D10 hybridized to BWS cell line B23.1, with a balanced (11;12) translocation. Signals are visible on the normal chromosome 11 and both derivative 11 and derivative 12 chromosomes, indicating that the YAC spans the breakpoint.

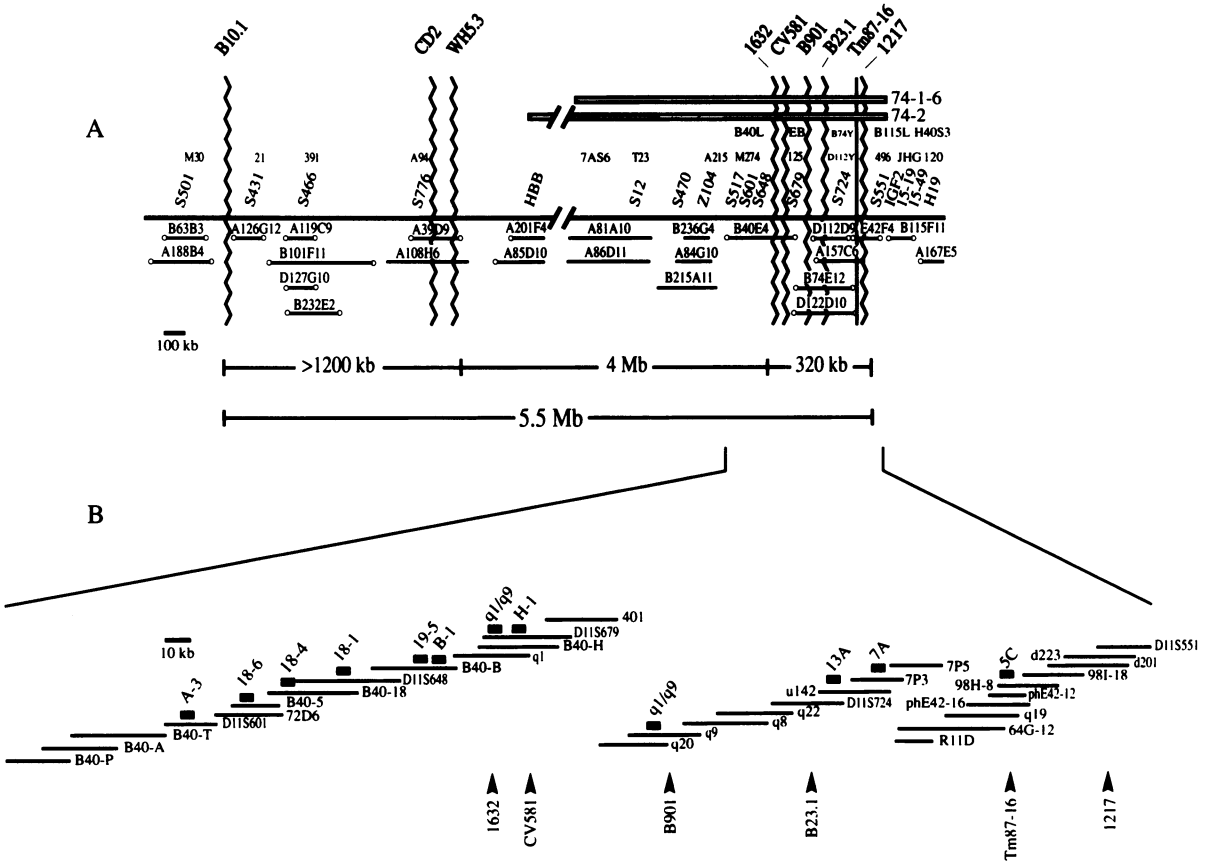


FIG. 2. Identification of a BWS/tumor-suppressor coincident region. (*A*) YACs spanning BWS translocation breakpoints. Vertical zigzag lines represent BWS balanced rearrangement breakpoint sites. Vertical shaded line represents the rhabdoid tumor cell line breakpoint. The open horizontal bars represent the rhabdomyosarcoma cell line RD-suppressing STFs 74-1-6 and 74-2, and the cluster of five BWS breakpoints and rhabdoid tumor breakpoint are contained entirely within them. Solid horizontal lines represent individual YACs. Circles on YACs indicate end clones. Probes and STSs are as indicated. Orientation is centromeric to telomeric. (*B*) A complete cosmid contig through the BWS/tumor-suppressor coincident region. Arrowheads denote BWS and rhabdoid tumor breakpoint sites and are positioned below the cosmids determined to span them. Boxes represent conserved sequences that detect transcripts.

and three additional breakpoints centromeric to them. One YAC, A39D9, spanned two of these latter breakpoints: an inversion of 11 in cell line WH5.3 (Fig. 1*A*) and a balanced (10;11) translocation in cell line CD2. Thus these two breakpoints were separated by no more than 265 kb, the size of A39D9 (Fig. 2*A*). The breakpoint in cell line B10.1, with a balanced (4;11) translocation, was at least an additional 1.2 Mb centromeric to the breakpoint in WH5.3, based on the size of YACs that hybridized between them (Fig. 2*A*).

**Isolation of BWS Breakpoint Sites Within Cosmids.** The previous experiments with YACs suggested that five BWS breakpoints were clustered within a region of ≤700 kb. To determine the precise physical relationship among the breakpoints, as well as their relationship to tumor-suppressing STFs, a complete cosmid contig was constructed through this breakpoint cluster. Cosmid libraries were constructed from YACs that spanned BWS breakpoints, which were then used in FISH analysis to identify those that crossed each of the breakpoints. Thus, cosmid D11S724 showed signals on both the derivative 11 and derivative 12 chromosomes in cell line B23.1, indicating that D11S724 crossed the translocation breakpoint in this patient (data not shown). Similarly, cosmid d201, derived from YAC E42F4, spanned the (11;16) translocation breakpoint in cell line 1217; cosmid q25, derived from YAC A39D9, spanned the inversion breakpoint in cell line WH5.3; cosmid q1, derived from YAC B40E4, spanned the (9;11) translocation in cell line 1632; cosmid D11S679 spanned the inversion breakpoint in cell line CV581; and cosmid q9, derived from YAC D122D10, spanned the (11;22) translocation breakpoint in cell line B901 (Fig. 2*B*). The sizes of cosmids within an overlapping contig established a minimum and maximum physical distance of only 295–320 kb spanning this cluster of five BWS balanced rearrangement breakpoints (Fig. 2*B*).

BWS-related tumors and some normal tissue from BWS and tumor patients show abnormal imprinting of *IGF2* and altered DNA methylation of *H19* (5, 6, 26–28), and it was thus of interest to determine the distance between these genes and the BWS breakpoints. However, both YAC and cosmid libraries lacked clones bridging this gap. Thus, two-color FISH using cosmid d201, which crossed the most telomeric BWS breakpoint, and cosINS/IGF2, which encompassed insulin and *IGF2*, were hybridized *in situ* to extended DNA. The gap between the two cosmids was approximately 100 kb, based on the size of the cosmids (data not shown).

**Localization of a Rhabdoid Tumor Breakpoint Within a BWS Breakpoint Cluster.** The rhabdoid tumor cell line TM87-16 contains as its sole karyotypic abnormality a balanced translocation involving 11p15.5 (18). PFGE analysis had suggested that this breakpoint lies within 265 kb of a BWS translocation breakpoint (10); however, neither breakpoint has previously been isolated. Both the derivative chromosomes 11 and 22 were visualized by FISH with YAC E42F4, which also spanned the (11;16) translocation in BWS cell line 1217 (Fig. 2*A*). To determine the relative order of these breakpoints, a phage library was constructed from E42F4. Phage phE42-12 identified novel 15.0-kb *Eco*RI and 7.0-kb *Hin*dIII bands in DNA from the rhabdoid tumor cell line (Fig. 3*A*). While these fragments were seen in no normal samples, FISH with phage

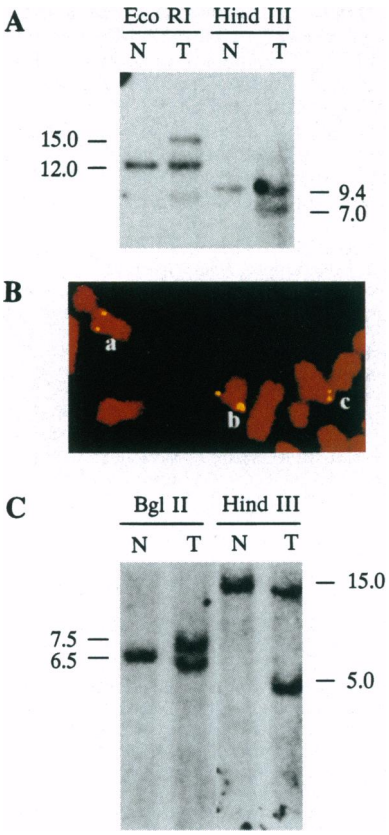


FIG. 3. Identification of phage and cosmid fragments spanning rhabdoid tumor and BWS translocation breakpoint sites. (*A*) Southern blot hybridization of a 12-kb *Eco*RI fragment from phE42-12, to normal tissue (N) and rhabdoid tumor cell line TM87-16 (T) DNA digested with *Eco*RI or *Hin*dIII. New fragments of 15.0 kb (*Eco*RI) and 7.0 kb (*Hin*dIII) are seen in the tumor line DNA. (*B*) FISH of phage phE42-12 to the rhabdoid tumor cell line. Signals are visible on the derivative chromosome 11 (a), normal chromosome 11 (b), and derivative chromosome 22 (c), confirming that the phage crosses this breakpoint. (*C*) A 150-bp *Eco*RI–*Pst* I subfragment from cDNA 13A hybridized to a Southern blot of DNA from normal (N) and BWS cell line B23.1, with a balanced (11;12) translocation (T), detecting new 7.5-kb (*Bgl* II) and 5.0-kb (*Hin*dIII) bands in patient DNA.

phE42-12 was also performed, to confirm that it crossed the breakpoint. Signals were apparent on both the derivative chromosome 11 and derivative chromosome 22 (Fig. 3*B*), confirming that phE42-12 contained the breakpoint. Finally, an ordered cosmid contig was constructed from cosmids derived from YACs E42F4 and A157C6, establishing that the rhabdoid tumor breakpoint lay within the BWS translocation breakpoint cluster, 100 kb telomeric and 30 kb centromeric to the two nearest BWS breakpoints (Fig. 2*B*).

**Coincidence of BWS Breakpoints and Tumor Suppressing STFs.** To determine whether any of the BWS breakpoints corresponded to the tumor suppressor region defined by STFs, DNA from the two smallest tumor-suppressing STFs was hybridized with cosmid clones that crossed each of the breakpoints. All five cosmids spanning breakpoints within the cluster of five BWS breakpoints hybridized to STFs 74-2 and 74-1-6, as did the phage spanning the rhabdoid tumor breakpoint. In contrast, cosmid q25, which spanned the inversion in cell line WH5.3 (the most telomeric of the group of three more centromeric breakpoints), did not hybridize to either STF, indicating that the more centromeric BWS breakpoints were excluded from the tumor-suppressing STFs. We refer to the more telomeric cluster of five BWS breakpoints, rhabdoid tumor breakpoint, and tumor-suppressing STFs as the BWS/tumor-suppressor coincident region.

**Identification of Transcripts.** Eleven conserved sequences that detected transcripts on Northern blots were identified within the BWS/tumor-suppressor coincident region (Table 1). Seven of these, A-3, 18-4, B-1, H-1, 7A, 13A, and 5C, detected transcripts in tissues susceptible to malignancy in BWS (Table 1). Two were apparently rearranged in BWS cell lines. Thus, the same 6.7-kb transcript was detected by cosmids q1 and q9, which lay on opposite sides of the (9;11) translocation and inversion in cell lines 1632 and CV581, respectively (Fig. 2*B*). In addition, clone 13A, isolated by direct YAC screening with YAC D122D10, appeared to detect the (11;12) translocation in cell line B23.1, displaying restriction fragments not seen in digests from 50 normal individuals (Fig. 3*C*). 13A also detected a 6.7-kb transcript (Table 1), suggesting that a large cDNA crosses multiple BWS breakpoints.

Of particular interest, the gene $p57^{KIP2}$, a cyclin kinase inhibitor, was recently isolated (29, 30), mapped to 11p15.5 by Matsuoka *et al.* (29), and therefore, proposed as a candidate tumor suppressor gene. By using the $p57^{KIP2}$ cDNA, we mapped the location of $p57^{KIP2}$ relative to the STFs, YACs, and cosmid contig and found that it was identical in location to B-1. $p57^{KIP2}$ was in the BWS tumor-suppressor coincident region, only 100 kb centromeric to BWS breakpoint 1632 (oriented 5′–3′ toward the telomere). $p57^{KIP2}$ transcripts corresponded to those identified by B-1 (Table 1).

## DISCUSSION

These experiments have four implications. (*i*) We have isolated a complete cosmid contig spanning 320 kb and including five BWS balanced germ-line rearrangement breakpoints and a balanced translocation from a rhabdoid tumor. This is less than

Table 1. Conserved sequences that detect transcripts

| Clone | Tissue pattern of expression | Transcript size, kb | Identification | Other |
|---|---|---|---|---|
| A-3 | Skeletal muscle, pancreas, placenta | 5.0 | CS | |
| 18-6 | Thymus, prostate | 4.0, 6.0 | CS | |
| 18-4 | All tissues, highest in liver, kidney | 2.5 | CS | |
| 18-1 | Colon, placenta, prostate | 1.0 | CS | |
| 19-5 | All adult tissues | 2.0 | CS | |
| B-1 | Brain, heart, kidney, skeletal muscle | 1.0, 2.0 | CS | $p57^{KIP2}$ |
| H-1 | Liver, fetal kidney, fetal heart | 1.4 | CS | |
| q1/q9 | All adult tissues, fetal liver | 1.0, 6.7 | CpG | Rearranged in BWS |
| 7A | Placenta, liver, all fetal tissues | 6.5, 6.7 | YAC | |
| 13A | All tissues, high in kidney, liver | 2.5, 6.7 | YAC | Rearranged in BWS |
| 5C | Skeletal muscle, fetal tissues | 0.8, 1.5 | YAC | Rearranged in rhabdoid |

CS, conserved sequence; CpG, CpG island; YAC, YAC hybridization.

half the size suggested by indirect PFGE mapping of three BWS breakpoints (10). Furthermore, the breakpoint cluster coincides with overlapping STFs containing a tumor suppressor gene. This BWS/tumor suppressor coincident region could thus represent a single large gene.

(*ii*) These BWS translocation breakpoints are closer to *IGF2* than was previously believed, within a distance over which positional effects might occur (31). Thus, the translocation breakpoints might influence the imprinting of genes near to but not spanning the breakpoints themselves, such as, on the telomeric side, *IGF2*, and on the centromeric side, $p57^{KIP2}$.

(*iii*) Two additional BWS balanced translocation breakpoints were isolated 4.0 and 4.2 kb centromeric to the BWS/tumor-suppressor coincident region, and a third was localized an additional 1.0 Mb centromeric to these two. We do not believe that a single gene could span all of the balanced rearrangements, as it would encompass ≥5.5 Mb and include the entire β-globin gene cluster within an intron. The simplest explanation to account for all of these data is that the BWS breakpoints represent more than one gene. The clinical features of BWS patients with translocations from the two breakpoint regions exhibited discernible differences, suggesting that disruption of different genes could give rise to distinct features of BWS and may be important in predicting the risk of developing tumors. Furthermore, Wilms tumors show LOH in both BWS breakpoint regions, while the common region of overlap of LOH in rhabdomyosarcoma, hepatoblastoma, and adrenocortical carcinoma does not include the more centromeric breakpoint region (32).

(*iv*) We have identified 11 transcripts by using fragments from cosmid and phage contigs through the BWS/tumor-suppressor coincident region. Several of these were expressed in tissues susceptible to malignancies associated with BWS and one of these, B-1, corresponded to the $p57^{KIP2}$ gene, previously suggested as a candidate BWS gene based on its location (29). Precise localization of $p57^{KIP2}$ to the BWS/tumor suppressor coincident region considerably increases the likelihood of its involvement in human cancer. This gene is particularly interesting because it is related to $p21^{CIP1/WAF1}$, a potential mediator of p53 tumor suppression (33, 34), and is itself a strong inhibitor of cyclin D- and E-dependent kinases (29, 30). Cyclin D1 appears to be important in several malignancies, including parathyroid adenoma, centrocytic lymphoma, and breast and squamous cell carcinomas (for review, see ref. 33).

Clearly, more than one gene must be involved in the pathogenesis of BWS and BWS-related tumors, as the tumor suppressor gene region defined by STFs contains one cluster of BWS breakpoints but excludes the three most centromeric breakpoints, as well as *IGF2* and *H19*, and the BWS/tumor suppressor coincident region also contains multiple genes. Most likely, a group of cancer-related genes fall within a several megabase region, similar to 1p, 3p, and 9p. It should be interesting to learn whether these genes are coordinately regulated or whether their relative proximity is accidental. Even if the latter is true, regional genetic changes in cancer such as LOH or loss of imprinting could have varying effects depending on the genes that are involved. A tantalizing idea is that the BWS translocation breakpoints have long-range cis-acting effects on genes such as *IGF2* and $p57^{KIP2}$. While this is a comparatively new idea in human genetics, the relative distances within the BWS/tumor suppressor coincident region fall within a range defined by positional effects seen in other species, such as telomere-influenced gene silencing in yeast and position effect variegation in *Drosophila* (31).

We thank T. Triche, N. Niikawa, A. Read, and O. Privitera for patient material; T. Cremer, A. Jauch, P. Meltzer, and M. Jakobs for assistance with FISH; Y. Nakamura for probes; G. Randhawa and J. Thompson for helpful discussions; and J. Patey for preparing the manuscript. This work was supported by National Institutes of Health Grant CA54358 (A.P.F.), March of Dimes Grant 6-FY94-0742 (A.P.F.), European Community Grant SCI0469 (P.L.), Netherlands Office of Scientific Research Grant 504-111 (M.M.), and National Institutes of Health Grant AG11085 (S.J.E.).

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