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Genetics

Isolation of molecular probes associated with the chromosome 15 instability in the Prader–Willi syndrome

(microdeletion/recombinant phage library/genetically unstable inserts/inverted repeats)

T. A. DONLON*, M. LALANDE*†, A. WYMAN‡, G. BRUNS*, AND S. A. LATT*§

*Mental Retardation Center, Division of Clinical Genetics, The Children's Hospital and Department of Pediatrics, Harvard Medical School, and §Department of Genetics, Harvard Medical School, Boston, MA 02115; and ‡Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

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ABSTRACT **Flow cytometry and recombinant DNA techniques have been used to obtain reagents for a molecular analysis of the Prader–Willi syndrome (PWS). *Hin*dIII total-digest libraries were prepared in λ phage Charon 21A from flow-sorted inverted duplicated no. 15 human chromosomes and propagated on recombination-proficient (LE392) and recBC⁻, sbcB⁻ (DB1257) bacteria. Twelve distinct chromosome 15-specific probes have been isolated. Eight localized to the region 15q11→13. Four of these eight sublocalized to band 15q11.2 and are shown to be deleted in DNA of one of two patients examined with the PWS. Heteroduplex analysis of two of these clones, which grew on DB1257 but not on LE392, revealed stem–loop structures in the inserts, indicative of inverted, repeated DNA elements. Such DNA repeats might account for some of the cloning instability of DNA segments from proximal 15q. Analysis of the genetic and physical instability associated with the repeated sequences we have isolated from band 15q11.2 may elucidate the molecular basis for the instability of this chromosomal region in patients with the PWS or other diseases associated with chromosomal abnormalities in the proximal long arm of human chromosome 15.**

The Prader–Willi syndrome (PWS) is a sporadic, genetic disorder characterized by hypotonia, hypogonadism, hypopigmentation, mental retardation, and obesity that affects ≈1/25,000 newborn individuals (1). Although neither the genetic nor the biochemical basis for this disease has been delineated, deletions or rearrangements at 15q11.2 have been reported to occur in at least half of the PWS patients examined cytogenetically (2–6).

The most common chromosome aberration in PWS patients is a deletion of band 15q11.2 (2); however, the size of these deletions is variable and presumably some are undetectable by using current cytogenetic techniques (see Fig. 1). Other aberrations at 15q11.2 occur in 2–5% of PWS patients (6–8), including translocations and duplications (both tandem and inverted), the latter termed inv dup(15) chromosomes. This multiplicity of chromosome rearrangements involving 15q11→13 in the PWS suggests that the proximal long arm of chromosome 15 contains DNA sequences, such as tandem or inverted repeats, which could predispose this segment to structural instability.

To define this region of the human genome on a molecular level, several DNA segments from the proximal long arm of chromosome 15 were isolated from λ phage Charon 21A libraries constructed from *Hin*dIII-digested DNA obtained from flow-sorted (9) inv dup(15) chromosomes. Because these inverted duplicated chromosomes contain two copies of all sequences within 15pter→15q13, we were able to more easily isolate DNA segments from the proximal long arm of chromosome 15. Using different cloning and insert screening strategies we determined that many of these sequences are genetically unstable in a recombination-proficient bacterial strain. Electron micrographs of heteroduplexes showed that unstable clones contain inverted repeats, which suggests at least one possible mechanism for their instability. In total, we describe in this report 11 different cloned DNA segments that map to 15q11→13; 4 of 8 tested are deleted in one of two PWS patients with a deletion of 15q11.2. These data suggest that there is molecular heterogeneity between 15q11 deletions in different PWS patients and that the cytogenetic instability of band 15q11.2 might be explained in terms of the types of DNA sequences it contains.

MATERIALS AND METHODS

Cell Lines. The lymphoblastoid cell lines used were from a karyotypically normal (46,XY) individual (MD-11), two patients with the PWS and different-sized deletions in 15q11.2 (DON-5, and DON-10), and two individuals with inv dup(15) chromosomes of different size [ALD-6 (10) and ALD-24 (11)] (Fig. 1).

Bacterial Strains. Strain DB1257 is related to strain DB1170 (12) and was constructed by P1 transduction of CES 200 (13) to kanamycin resistance using a lysate prepared on NK 5857 (*Trp::Tn5 SupF 58*). The partial genotype of CES 200 is recBC⁻, sbcB⁻. Strain LE392 has been described (14).

Construction of the inv dup(15) Library. Charon 21A libraries were created from 50 ng of *Hin*dIII-digested DNA from 6 million flow-sorted, inv dup(15) chromosomes, isolated from cell line ALD-24. Chromosomes were sorted by using a Becton Dickinson dual-beam fluorescence-activated chromosome sorter (FACS IV) and Hoechst 33258 plus chromomycin A_3 as fluorochromes (11). The inv dup(15) is described as 15(pter→q13::q13→pter). In the present work, 40,000 plaque-forming units (pfu) were propagated on LE 392, whereas 10,000 pfu were propagated on DB1257.

A separate library was constructed from *Hin*dIII-digested genomic DNA from the cell line ALD-24 and it was propagated on strain DB1257.

Screening of Libraries. Phage from the LE 392-grown library with inserts containing repeated human sequences were detected by multiple rounds of Benton–Davis screening (15), using radiolabeled human DNA as probe, and inserts lacking repeated sequences were selected for further analysis using Southern blot hybridization (16). Some phage from the DB1257-grown library were processed directly, without preliminary Benton–Davis screening. Other phage from the DB1257-grown library were picked onto lawns of LE392 and

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Abbreviations: PWS, Prader–Willi syndrome; pfu, plaque-forming units; kb, kilobase(s); bp, base pair(s).

†Present address: National Research Council, Biotechnology Research Institute, Montreal, Canada.

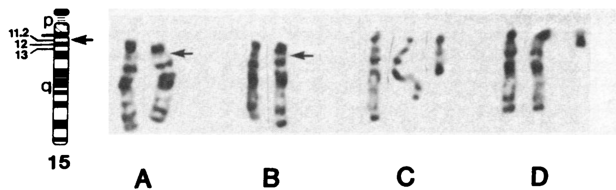


FIG. 1. Sets of no. 15 chromosomes from four individuals, including structural abnormalities, used in the present study. To the extreme left is a standard ideogram of chromosome 15. In each set, the abnormal chromosome is at the right and the location of the deletion is denoted by an arrow. DON-5 (*A*) and DON-10 (*B*), both with 15q11.2 deletions, the latter somewhat larger than the first. ALD-24 (*C*) and ALD-6 (*D*), both with inv dup(15) chromosomes, in addition to two normal homologs, with breakpoints in the inv dup(15) at 15q13 and 15q11.1, respectively.

DB1257 (12), and phage growing poorly on LE392, or not at all, were selected for further analysis.

Radiolabeling of Probes and Prehybridization. Phage inserts were ^{32}P-labeled for hybridization to Southern blots by using either T4 DNA polymerase (17) or the random primer method as described by Feinberg and Vogelstein (18). Those radiolabeled probes that were known to contain high-copy-number repeats were treated, prior to hybridization, to minimize nonspecific background (19, 20). To accomplish this, probes were precipitated in ethanol, in the presence of 40 μg of tRNA per 200 μl, and resuspended in 70 μl of 1 M phosphate buffer (pH 6.8) and 500 μl of a 2.5 mg/ml stock of sheared human placental DNA. The samples were then boiled for 10 min, incubated at 65°C for 4–8 hr, mixed with prehybridization solution, and added to the filter bags containing the Southern blots. DNA hybridization to nitrocellulose blots (16) and washing were as described (21).

Radiolabeling with ^{3}H for *in situ* hybridization was also performed by using the random primer method, with [^{3}H]dATP, [^{3}H]dCTP, and [^{3}H]dTTP, resulting in probes with $>10^{8}$ cpm/μg. Prehybridization with sheared, human genomic DNA was also necessary for *in situ* hybridization with some of these probes.

Chromosomal Mapping. Initial mapping of single-copy inserts was accomplished by using DNA from Chinese hamster–human somatic cell hybrid cell lines containing various human chromosomes, which have been described by Bruns *et al.* (22). These lines and their corresponding chromosomes are G35D3 (2p,4,7,8,9,10,14,16,17,19,20,21,22); G35D5 (1,2,3,6,7,10,13,14,15,16,17,18,19,20,22); and G35E4 (4p−q−,9,15,19). Probes hybridizing to cell lines G35D5 and G35E4, which contain chromosome 15 as their only common human chromosome, but not to G35D3 were tentatively assigned to human chromosome 15.

Putative chromosome 15-specific inserts were then mapped more precisely by using *in situ* hybridization (23). Autoradiographic localization of the hybridized probe was performed by using simultaneous fluorescent R-banding/transmitted light (24).

Electron Microscopy. The secondary structures of phage inserts were examined by electron microscopy of heteroduplexes formed between Charon 21A, containing the human DNA inserts, and Charon 30::Tn*10*Δ (12), which contains a Tn*10* transposable element inserted 3.2 kilobases (kb) to the left of the *Hin*dIII site used for cloning in Charon 21A. Tn*10* is visualized as a 2.7-kb loop with a 70-base-pair (bp) inverted repeat at its site of insertion that can be used to confirm the formation of a Charon 21A/Charon 30 heteroduplex and also to serve as an internal size standard. Preparations were hybridized at 37°C in 50% formamide for 35 min, spread on a hypophase of 20% formamide (25), and coated with cytochrome *c* (26). Preparations were then shadowed with Pd/Pt and examined with a JEOL 100S electron microscope at a final magnification of 4000–10,000×.

RESULTS

A three-dimensional fluorescence histogram of the smaller human metaphase chromosomes from the lymphoblastoid cell line ALD-24, including the inv dup(15) chromosome used for DNA isolation and library construction, is shown in Fig. 2.

Two recombinant phage libraries were constructed from *Hin*dIII-digested inv dup(15)-enriched DNA inserted into the *Hin*dIII site of Charon 21A. The first, yielding 40,000 pfu from 40 ng of DNA, was propagated on the bacterial host LE392. The second, yielding 10,000 pfu from 10 ng of DNA, was propagated on the host DB1257. Three sets of phage inserts from these two libraries were then analyzed. The first set was derived from phage propagated on LE392, which had been tested for homology with highly repeated human DNA by two Benton–Davis screenings followed by a Southern blot in which the phage insert was used to probe genomic DNA. In this set only repeat negative inserts were examined. Of 68 phage inserts tested, 24 were sufficiently free of repeated sequences that chromosome mapping was possible (Table 1). Seven of 24 mapped were given an initial assignment to chromosome 15 based on blotting inserts to digested DNAs from human–rodent somatic cell hybrid lines. An example is shown in Fig. 3.

Three of the seven chromosome 15-specific inserts were mapped more precisely by using *in situ* hybridization; two single-copy inserts mapped to 15q11→13 and one moderately repeated insert mapped to 15p11. Fig. 4 shows a histogram of grain localizations over the proximal long arm, using pML34, a single-copy insert, as probe.

Four of the seven chromosome 15-specific inserts, including pML34, appeared to be single-copy, thus permitting them to be subchromosomally mapped by comparative hybridization intensity to DNAs from cell lines aneusomic for portions of chromosome 15, such as those depicted in Fig. 1. All four of these mapped to 15q11→13.

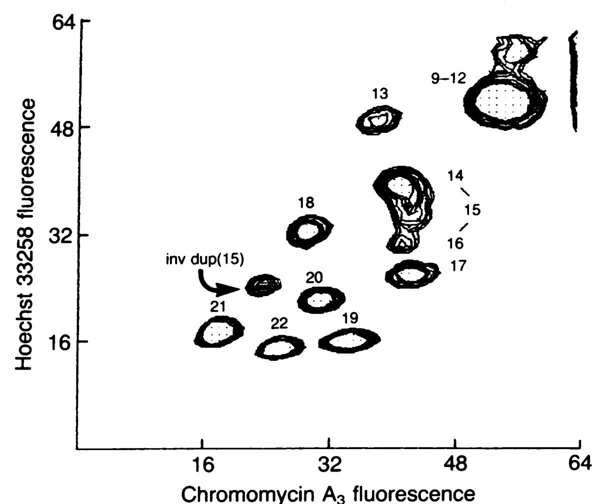


FIG. 2. Bivariate flow histogram of cell line ALD-24 showing the distribution of the smaller metaphase chromosomes. The chromosomes were isolated and stained with a combination of Hoechst 33258 (ordinate) and chromomycin A_3 (abscissa). Contour lines correspond to the relative numbers of chromosomes. The peak marked inv dup(15), as judged from previous analyses (11), was used for chromosome sorting.

Table 1. Characterization of cloned DNA inserts from inv dup(15) phage library

Conditions	Plaques, no.		Mapping results				
	Examined	Mapped*	Chromosome 15†	15q11→15q13‡	15p11§	15q11.2¶	% on chromosome 15
LE 392, negative ×2, B–D	68	24	7	4	1	2	29
DB1257, random	15	12	3	NT	2	0	25
DB1257, selective‖	7	7	4	4	0	2	57

NT, not tested; B–D, Benton–Davis screening.
*Using prehybridized blots when necessary.
†Using hybrids.
‡Quantitative dosage blotting and/or *in situ* hybridization.
§*In situ* hybridization.
¶Deleted in DON-10 (Fig. 1*B*).
‖Host-limited (i.e., propagates well on DB1257 but not on LE392).

To determine if any of these probes is reduced in copy number in DNA from patients with the PWS, comparative hybridization intensity was assessed (21) by using DNA from two patients with PWS who had visible deletions of 15q11.2 (Fig. 1). Fig. 5 shows the results for the 2.2-kb probe, pTD3-21; H2-26 is a 3.6-kb chromosome 13-specific probe (28) used as an internal standard. Examination of this blot shows that, of the DNA samples from the two PWS patients, hybridization to that of DON-10 is reduced in intensity, strongly indicating that this probe is deleted from one homologue of this patient. As expected, there is increased hybridization of this probe to ALD-24 DNA, which should contain four copies per genome, but normal hybridization to DNA from ALD-6, a cell line containing a smaller inv dup(15) and only two copies of 15q11.2 per genome.

Additional phage were propagated on DB1257 in an attempt to retain inserts possibly unstable if cloned in LE392 (12). Of an initial group of 15 such inserts chosen at random, 12 could be mapped. Three of these, 1 containing highly repetitive sequences and 2 containing moderately repeated sequences, localized, by means of hybrid cell DNA mapping panels, to chromosome 15 (Table 1). The moderately repeated probes appear to be identical, as determined by insert size and hybrid mapping pattern, to the repeated probe from the LE392 library that had been mapped by *in situ* hybridization to band 15p11.

Seven additional phage from the DB1257 library were identified because they would not propagate well on LE392. All seven could be mapped; four of these, one single-copy and three containing highly repeated sequences, were localized to chromosome 15 by hybrid DNA panels and to 15q11→13 by quantitative DNA blot hybridization (Table 1). Of these four, two showed a reduced copy number in the DNA from DON-10, indicating their localization within band 15q11.2, which is deleted in DON-10.

These results suggest not only that the PWS is composed of a heterogeneous class of deletions—e.g., the deletions in DON-5 and DON-10 clearly differ—but also that a portion of the DNA segments from the proximal long arm of chromosome 15 is unclonable in recombination-proficient hosts and, therefore, is genetically unstable. To determine if the inv dup(15) contains a higher proportion of genetically unstable sequences, relative to the genome as a whole, the fraction of host-limited inserts from the inv dup(15) library was compared with inserts isolated from a total genomic library, isolated under identical conditions and propagated on DB1257. Only 3.4% (17/500) of the inserts from the total genomic library failed to grow on LE392, as compared to 7.1% (40/560) from the inv dup(15), reinforcing the suspicion that the proximal long arm of chromosome 15 is enriched for genetically unstable DNA segments.

Based on the evidence of Wyman *et al.* (12), who have shown that the majority of "host-limited" phage contains palindromic or inverted repeated DNA segments, we tested whether our host-limited clones had similar structures. Heteroduplex analyses of the two phage with inserts that map to 15q11.2, λ IR-4 and λ IR-10, showed that these inserts contain small (≈300 bp) inverted repeated segments (Fig. 6). Restriction map and DNA blot data (Fig. 7) show these segments to lie within sections of the inserts containing highly repeated DNA, analyzed by blotting the multienzymatically digested DNAs and hybridization with radiolabeled BLUR-8 DNA (29).

DISCUSSION

The present study identifies molecular probes deleted in no. 15 chromosomes from a patient with the PWS. Since these

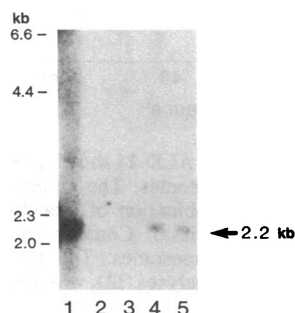


FIG. 3. Location of probe pTD3-21 to human chromosome 15 using a rodent–human somatic cell hybrid DNA mapping panel. DNA digested with *Hin*dIII endonuclease: 46,XY (lane 1), Chinese hamster (lane 2), hamster–human hybrids G35D3, G35D5, and G35E4 (lanes 3, 4, and 5, respectively), the latter two of which have only chromosome 15 as their common human chromosome.

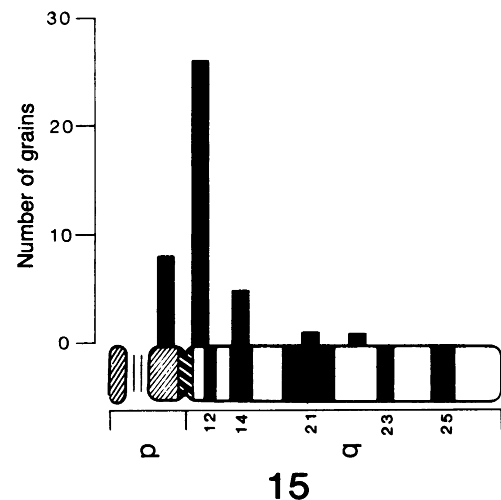


FIG. 4. Localization of probe pML34 to 15q11→15q13 by *in situ* hybridization. Of 100 metaphase cells examined, 37 had grains over 15q11→13, and of 159 total grains, 24.5% were over this region, with no other chromosomes exhibiting hybridization significantly above background.

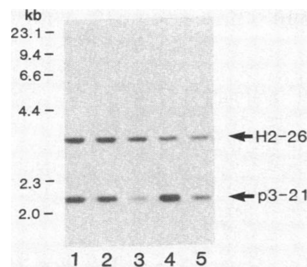
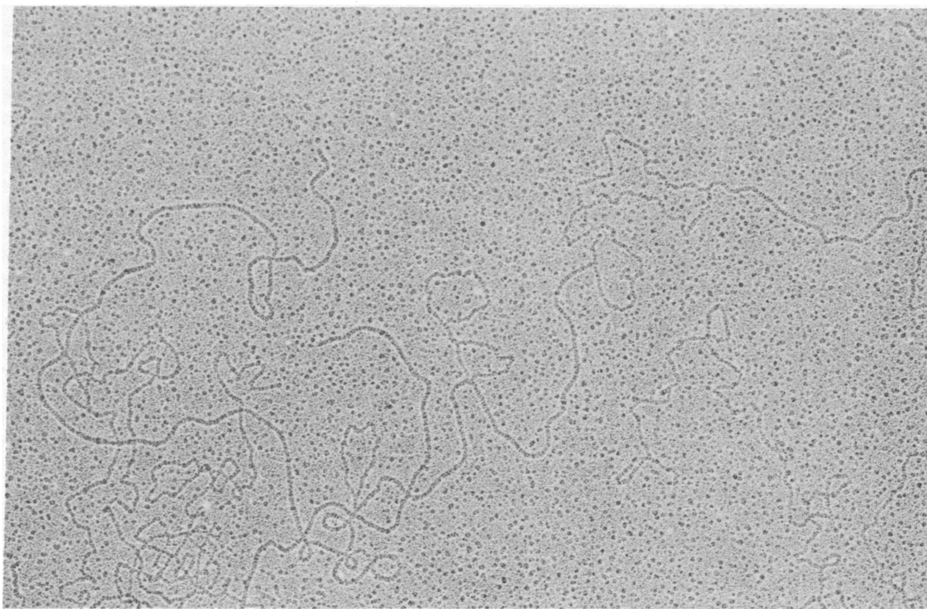


FIG. 5. Localization of probe pTD3-21 to the deleted segment of 15q11.2 in cell line DON-10 by using quantitative DNA blotting. Each lane contains 1.5 μg of DNA, as assayed fluorometrically (27). Samples were digested with *Hin*dIII endonuclease, and representative samples were obtained from 46,XX (lane 1), DON-5 (lane 2), DON-10 (lane 3), ALD-24 (lane 4), and ALD-6 (lane 5) cells. Reduced hybridization of the 2.2-kb band in lane 3 (DON-10) indicates its deletion from one homologue in this cell line. Probe H2-26, a 3.6-kb insert from chromosome 13, was used as an internal standard for hybridization intensity.

probes are not deleted in the DNA from another PWS patient, who also has a cytologically visible deletion, the results suggest the heterogeneity of 15q1 deletions in the PWS. Of greater significance, the recombinant library described and the probes so isolated now permit a molecular analysis of PWS deletions to refine previous cytological diagnoses.

Different strategies for isolating DNA segments deleted in PWS patients produced differential enrichments for, and possibly a differential representation of, segments distributed over the proximal long arm of chromosome 15. By identifying phage containing inserts that grow poorly on a recombination-proficient bacterial host (LE392), it was possible to enrich for segments from band 15q11.2. Characterization of additional phage will help establish the quantitative significance of this observation. Surprisingly, the poorest representation of chromosome 15-specific probes was obtained by isolating phage that were human-repeat negative, a strategy that has worked successfully for the isolation of DNA segments from other flow-sorted chromosome libraries. The lack in success of this screening strategy may be explained by (*i*) the



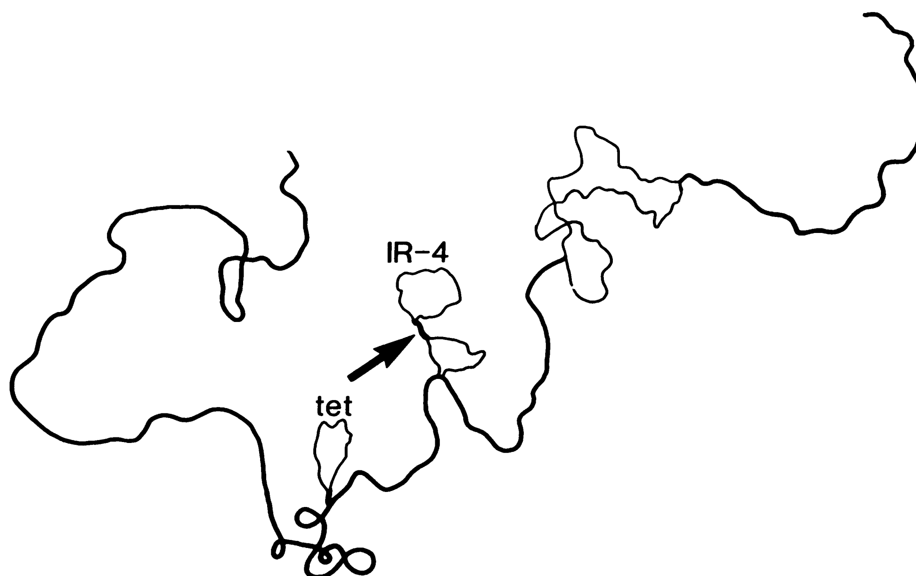


FIG. 6. Electron micrograph of a heteroduplex formed between λ IR-4, containing a 5.0-kb insert, and Charon 30, containing tet, a 2.7-kb Tn*10* insertion 3.2 kb to the left of the *Hin*dIII site. The inverted repeat found in λ IR-4 (shown by the arrow) is ≈300 bp in length.

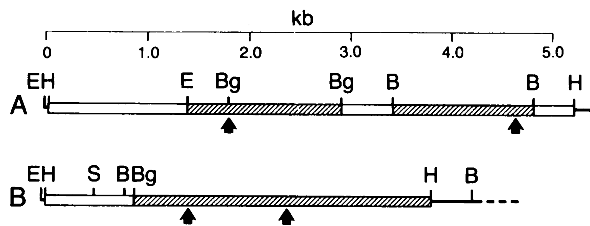


FIG. 7. Restriction maps of λ IR-4 (*A*) and λ IR-10 (*B*) inserts, showing the locations of BLUR-8-homologous restriction fragments (hatched) relative to the segments shown to be involved in the inverted repeated, secondary structure (arrows) (e.g., Fig. 6). E, *Eco*RI; H, *Hin*dIII; Bg, *Bgl* II; B, *Bam*HI; S, *Sal* I.

relatively high proportion of moderately repeated DNA sequences in the inv dup(15), which may not be identified by the Benton–Davis screenings, and (*ii*) an abundance of highly repeated DNA on the proximal long arm of chromosome 15, which would be eliminated by this screening procedure. Therefore, it may be possible to enrich for segments specific to the proximal long arm by screening for highly repeated sequences. The secondary structure of several host-limited phage inserts was examined with the goal of determining the causes for the poor growth of phage containing them on recombination-proficient hosts. Two inserts examined, IR-4 (Fig. 6) and IR-10 (not shown), demonstrated the presence of inverted repeats of about 300 bp in length, consistent with their belonging to the *Alu* family (30). Fig. 7 orients the inverted repeats, seen in the electron micrographs, with respect to the restriction maps for these inserts; segments containing homology to BLUR-8 (29) are marked by hatching, and the areas involved in the stem structure are identified by arrows.

Inverted repeat elements could conceivably explain many of the deletions and rearrangements involving band 15q11.2 in PWS. The 15q11.2 deletion could result from unequal sister-chromatid exchange or simple looping out of DNA, mediated by direct or inverted repeats. Fraccaro *et al.* (6) have evidence compatible with this latter model and they describe a PWS patient with a small segment of chromatin in his cells that is compatible in size with its being derived from 15q11.2. The inv dup(15), less frequently seen in the PWS, has been postulated to occur by a U-type crossover during meiosis, primarily in the female (31). This could easily be accomplished by homologous pairing between two repeats in opposite orientation, followed by DNA-strand interchange. Tests of these various models could benefit from the availability of polymorphic DNA probes (32) of the type that could be isolated from a chromosome 14- plus chromosome 15-enriched library recently prepared in our laboratory.

The exact role that 15q11.2 deletions and translocations play in the cause of the PWS is poorly understood. For example, patients have been described who have deletions, translocations, inverted duplication, and apparently normal no. 15 chromosomes, indicating that dosage for 15q11.2 may not be the sole cause for this disease. Additionally, grossly normal no. 15 chromosomes in PWS patients may have duplications of some regions and deletions of others. Deletions of 15q11.2 exist in other patients who do not have the PWS, three of whom we have studied (data not shown). By constructing a grid of evenly spaced, cloned DNA segments, it may ultimately be possible to correlate the loss of certain segments of human chromosome 15 with different clinical phenotypes.

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