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De Novo Truncation of Chromosome 16p and Healing with $(TTAGGG)_n$ in the α-Thalassemia/Mental Retardation Syndrome (ATR-16)

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Summary

We have previously described a series of patients in whom the deletion of 1–2 megabases (Mb) of DNA from the tip of the short arm of chromosome 16 (band 16p13.3) is associated with α-thalassemia/mental retardation syndrome (ATR-16). We now show that one of these patients has a de novo truncation of the terminal 2 Mb of chromosome 16p and that telomeric sequence $(TTAGGG)_n$ has been added at the site of breakage. This suggests that the chromosomal break, which is paternal in origin and which probably arose at meiosis, has been stabilized in vivo by the direct addition of the telomeric sequence. Sequence comparisons of this breakpoint with that of a previously described chromosomal truncation $(\alpha\alpha)^{TI}$ do not reveal extensive sequence homology. However, both breakpoints show minimal complementarity (3–4 bp) to the proposed RNA template of human telomerase at the site at which telomere repeats have been added. Unlike previously characterized individuals with ATR-16, the clinical features of this patient appear to be solely due to monosomy for the terminal portion of 16p13.3. The identification of further patients with "pure" monosomy for the tip of chromosome 16p will be important for defining the loci contributing to the phenotype of this syndrome.

Introduction

The duplicated human α-globin genes (αα/αα) lie at a close, but variable, distance (170–430 kb) from the tip of the short arm of chromosome 16 (16p13.3) (Breuning et al. 1987; Buckle et al. 1988; Wilkie et al. 1991). The proximity of the α-globin genes to the 16p telomere, together with the relative ease with which hemizygous deletions (– –/αα) can be identified (producing the hematologic phenotype of α-thalassemia trait; reviewed in Higgs et al. 1989), provides a useful model system for studying the mechanisms and consequences of terminal chromosome deletions.

We have described elsewhere eight patients with α-thalassemia and associated mental retardation who appear to have terminal deletions of 16p (the α-thalassemia/mental retardation syndrome involving structural abnormalities of chromosome 16 [ATR-16]). Molecular and cytogenetic analysis indicates that at least four of these patients have unbalanced karyotypes resulting from translocations in which the tip of chromosome 16p has been replaced by additional chromosomal material (Buckle et al. 1988; Lamb et al. 1989; Wilkie et al. 1990*a*). Analysis of the chromosomal breakpoints in these patients may be instructive for understanding the mechanisms by which sporadic translocations occur, but, because of the additional chromosomal aneuploidy, these patients are less informative for molecular dissection of the ATR-16 phenotype than are individuals with "pure" chromosome 16p monosomy.

In an earlier report we described a family segregating for an α-thalassemia determinant $(\alpha\alpha)^{TI}$ in which we demonstrated that a truncation of chromosome 16 had deleted sequences distal to the α-globin genes (Wilkie et al. 1990*b*). This chromosomal break was stabilized by the addition of telomeric repeat sequence $(TTAGGG)_n$. Individuals carrying the $(\alpha\alpha)^{TI}$ chromosome are of normal intellect, presumably because

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monosomy for 16p distal to α-globin (120–380 kb) has no major effect on development.

Here we describe a patient with a de novo terminal deletion of 16p, a deletion which is much larger (2 Mb) and which is also stabilized by telomeric repeat sequences. This patient is mentally handicapped (IQ = 53), indicating that hemizygosity for one or more genes in the deleted segment, proximal (centromeric) to α-globin, may adversely affect mental development. In addition to helping to localize a critical region for mental handicap in ATR-16, these results provide further information on the mechanism and sequence requirements for the healing of chromosomal breaks in vivo.

Material and Methods

Southern Blot Hybridization

DNA was prepared from venous blood, lymphoblastoid cell lines, or somatic cell hybrid lines. Standard methods were used for restriction-endonuclease digests, Southern blots, and hybridization with radiolabeled probes (Feinberg and Vogelstein 1983; Old and Higgs 1983). The probes GGG1/D16S259 (Germino et al. 1990), PNL56S/D16S145 (Harris et al. 1990), and α-globin 5′HVR/D16S262 (Jarman and Higgs 1988) have been described elsewhere. The cosmid cUW9 contains an ~35-kb genomic DNA insert generated by partial *Mbo*I digestion and inserted into the *Bam*HI site of C2RB (Blonden et al. 1989). The insert contains both GGG1 and PNL56S (fig. 1*c*). The probes Ps 0.6 and PsH 0.9 were developed during the present study. Hybridization with the latter probes, as well as with fragments of cUW9, was at 55°C in phosphate buffer (Church and Gilbert 1984), with sonicated, denatured, and unlabeled human DNA (400 μg/ml). Paternity was confirmed by fingerprint analysis of *Hin*fI-digested genomic DNA, by using the α-globin 3′HVR/D16S85 probe (Jarman et al. 1986) at low stringency (Wilkie et al. 1990*a*).

Pulsed-Field Gel Electrophoresis (PFGE)

DNA from Epstein-Barr virus (EBV)–transformed lymphocytes was isolated in agarose blocks and was digested with restriction endonucleases (New England Biolabs) according to the supplier's instructions. Separation was achieved by either field-inversion gel electrophoresis (FIGE) or contour-clamped homogeneous electric field electrophoresis (CHEF). FIGE was performed in a BRL H1 horizontal gel box controlled by the DNA Star Pulse system. Gels were run for 70 h at 150 V, with a 3:1 ratio of forward pulses to reverse pulses. The initial forward pulse was for 3 s and was ramped up to a final forward pulse of 50 s. CHEF gels were run using the Bio-Rad CHEF DRII system. Gels were run for 63.5 h at 110 V, with an initial pulse time of 20 s, which was ramped to a final pulse time of 50 s.

Somatic Cell Hybrids

The hybrid cell line J-BH1E was derived from the fusion of a lymphoblastoid cell line, derived from the patient (BO), with a mouse erythroleukemia (MEL) line, by the Deisseroth and Hendrick (1978) method as modified by Zeitlin and Weatherall (1983). It contains the normal chromosome 16 of BO. A modified protocol (W. G. Wood and J. Sharpe, unpublished data) was used to produce the mouse/human hybrid J-BH48, which contains the abnormal chromosome 16 of BO. Lymphoblastoid cells from the patient and an adenine phosphoribosyltransferase–negative ($APRT^-$) MEL cell line were allowed to adhere to a poly-L-lysine–coated flask, prior to fusion with 50% polyethylene glycol. Two hours after fusion, dikaryons were selected by sedimentation through a 10%–40% FCS gradient prepared in Hanks balanced salt solution (Gibco). After adhesion to a new flask, hybrids containing human chromosome 16 were obtained by selection for $APRT^+$ cells in medium containing methotrexate, adenine, and ouabain.

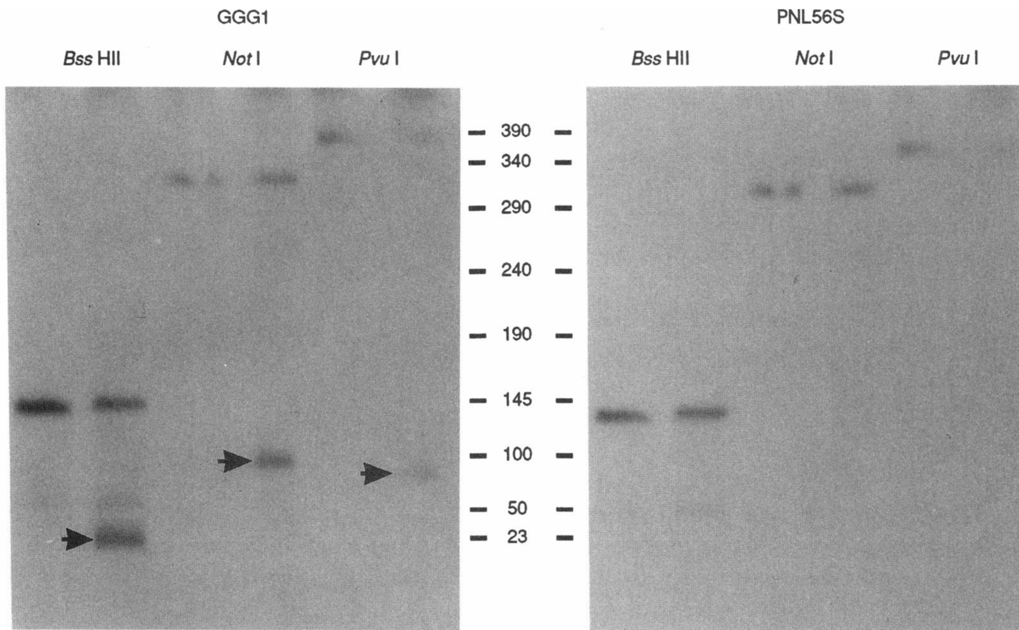
PCR and Sequence Determination

PCR was performed using the primers NB1 or NB2 with TEL1 (table 1). Amplification in a Perkin Elmer Cetus DNA Thermal Cycler used 1.5 units of AmpliTaq (Cetus) in 3 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris-Cl pH 8.8, 67 μM EDTA, 0.01% gelatin, 10 mM 2-mercaptoethanol, and 10% dimethylsulfoxide, with a denaturing temperature of 94°C for 1 min, an annealing temperature of 58°C for two cycles of 2 min, followed by 65°C for 29 cycles, and extension at 72°C for 3 min, apart from the last cycle, which was for 10 min. The product was digested at 37°C for 2 h with λ exonuclease (Gibco BRL) prior to direct sequencing (Higuchi and Ochman 1989) using primers NB1, NB2, or NB3 (table 1) with the Sequenase kit (USB). The fragment PsH 0.9, which overlaps the breakpoint, was introduced into M13, and normal sequence was obtained by the dideoxynucleotide chain-termination protocol (Sanger et al. 1977).

Results

Clinical Features

The proband (BO) was born to nonconsanguineous South African parents of Dutch extraction. His medical


a
GGG1
PNL56S
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Not I
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340
290
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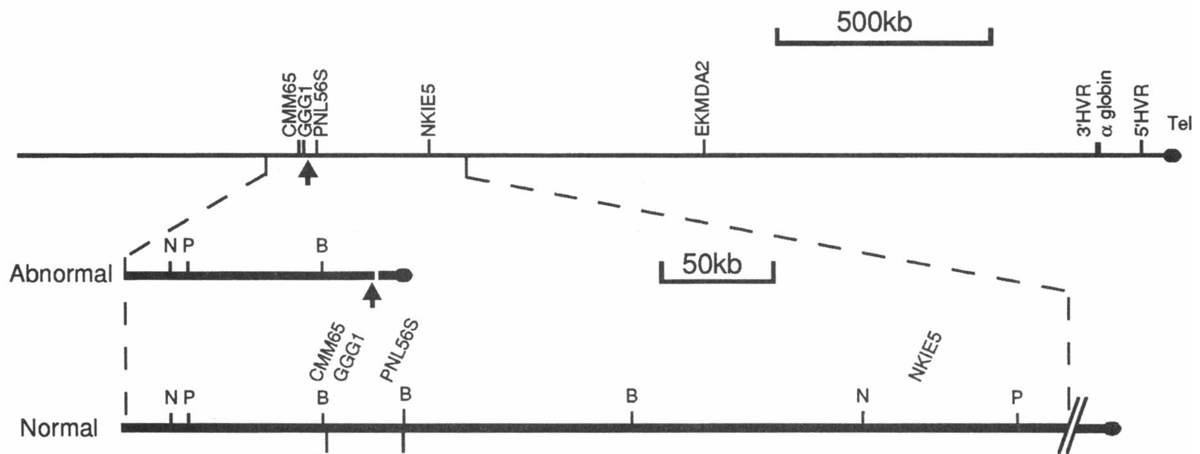
b
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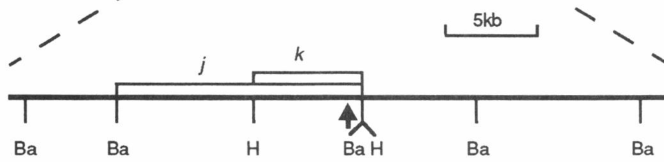
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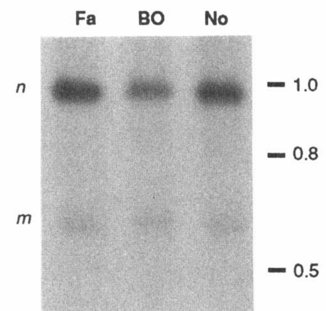
Fa
BO
No
n
m
1.0
0.8
0.5

Table 1

Primers Used in PCR and DNA Sequencing of the Breakpoint in BO

Primer[a]	Sequence
NB1	5′-CTCCTTGAACTTGAGCCCACG-3′
NB2	5′-TAAGCGAGCGGCTCCAAT-3′
NB3	5′-TATAACAAACACATCAGATG-3′
TEL1	5′-TATGGATCCCTAACCCTGACCCTAACCC-3′

[a] NB1, NB2, and NB3 are nested primers that lie proximal to the breakpoint. TEL1 contains a single-base mismatch (A→G) in the canonical C-strand telomere sequence and has a 7-bp clamp at the 5′ end, to increase the fidelity of the PCR reaction.

history has been fully documented elsewhere (Borochovitz et al. 1970; Bowcock et al. 1984; Wilkie et al. 1990*a*). He has hemoglobin H (HbH) disease (α-thalassemia) and is mentally handicapped, with an IQ of 53. Although the patient was originally reported to have a normal karyotype (Borochovitz et al. 1970; Bowcock et al. 1984), high-resolution cytogenetic analysis has revealed a subtle, but consistent, shortening of band 16p13.3 of one chromosome 16 homologue (Wilkie et al. 1990*a;* V. J. Buckle, personal communication). This abnormality was not detected in either parent.

Analysis of the α-Globin Genes

Hematologic indices and α-globin genotypes of all family members have been reported elsewhere (Bowcock et al. 1984). Southern blot analysis revealed a normal genotype (αα/αα) in the patient's father; the mother is a carrier for α-thalassemia trait and has the genotype -α/αα. The proband has the genotype --/-α, characteristic of HbH disease. Analysis of a hypervariable locus (α-globin 5′HVR) which lies 90 kb telomeric of the α-globin genes (Jarman and Higgs 1988) showed that the proband had inherited only one α-globin allele, which was linked to the maternal -α haplotype. These data show that the -- haplotype is due to a de novo deletion of the paternal α-globin gene complex (Wilkie et al. 1990*a*).

Mapping the Proximal Extent of the Deletion

The breakpoint of the deletion was previously shown to lie approximately 2 Mb proximal to the 16p telomere (Wilkie et al. 1990*a*), within the 24-kb interval separating the locus GGG1 and the more distal locus PNL56S. Further characterization of the abnormal chromosome was achieved by PFGE. Both normal and abnormal restriction fragments were detected when genomic DNA from BO was cleaved with *Mlu*I, *Not*I, *Bss*HII, or *Pvu*I and probed with GGG1 (Wilkie et al. 1990*a;* fig. 1*a*). The abnormal restriction fragments were not seen with PNL56S (fig. 1*a*), confirming that sequences detected by this probe are deleted from the abnormal chromosome 16 homologue. Comparison of the abnormal GGG1-specific fragments with the existing long-range map (Harris et al. 1990) showed that the distal end of each fragment terminates at the same position (fig. 1*b*). PFGE analysis of the patient's father showed no abnormality. These data indicate that a de novo deletion has resulted in the loss of approximately 2 Mb of DNA from the tip of the short arm of chromosome 16, an interpretation which is consistent with the cytogenetic observations.

Characterizing the Breakpoint

To define the breakpoint more accurately, the region spanning GGG1 and PNL56S, corresponding to the cosmid cUW9, was analyzed. The insert from cUW9 was cleaved with *Bam*HI to generate four fragments of 4 kb, 13 kb, 6 kb, and 9 kb (fig. 1*c*). Each was used to probe conventional Southern blots of DNA from the family, but no breakpoint bands were detected with five different restriction enzymes (data not shown).

Figure 1 Mapping the breakpoint on chromosome 16. *a,* PFGE analysis of normal and proband DNA digested with rare-cutter restriction enzymes. Abnormal bands were detected with the probe GGG1 (arrow) but not with the probe PNL56S, which lies distal to the breakpoint. In each pair, the normal DNA is on the left, and proband DNA is on the right. Faint bands detected, in both lanes, with *Bss*HII are the result of an intervening site which is resistant to complete digestion. Size markers are in kilobases. *b,* Distal region of chromosome 16p13.3, which is shown above restriction maps of the abnormal and normal chromosomes derived from PFGE analysis. The chromosomal breakpoint is identified by an arrow (↑). When the abnormal bands detected by GGG1 were mapped, they all terminated at the same distal position, suggesting that the end of the chromosome has been reached. PNL56S is absent from the truncated chromosome. N = *Not*I; P = *Pvu*I; and B = *Bss*HII. *c,* Map of the cosmid cUW9, showing the restriction fragments used to localize the breakpoint, which is indicated by an arrow (↑). Fragments *j* and *k* revealed an abnormal band of approximately 100 kb when they were used to probe *Not*I-digested patient DNA. Ba = *Bam*HI; and H = *Hin*dIII. *d,* Detail of *Hin*dIII insert of the plasmid pUW9.1 containing the breakpoint (↑). Fragments *m* (i.e., Ps 0.6) and *n* (i.e., PsH 0.9) were used to probe Southern blots of *Hin*dIII/*Pst*I-digested DNA from the proband (BO), his father (Fa), and a normal control (No). The reduced intensity of band *n* in the proband indicates that this fragment is absent from the deleted chromosome, although no breakpoint band was detected. The control fragment *m* appears in equal intensity in all samples. H = *Hin*dIII; and Ps = *Pst*I.

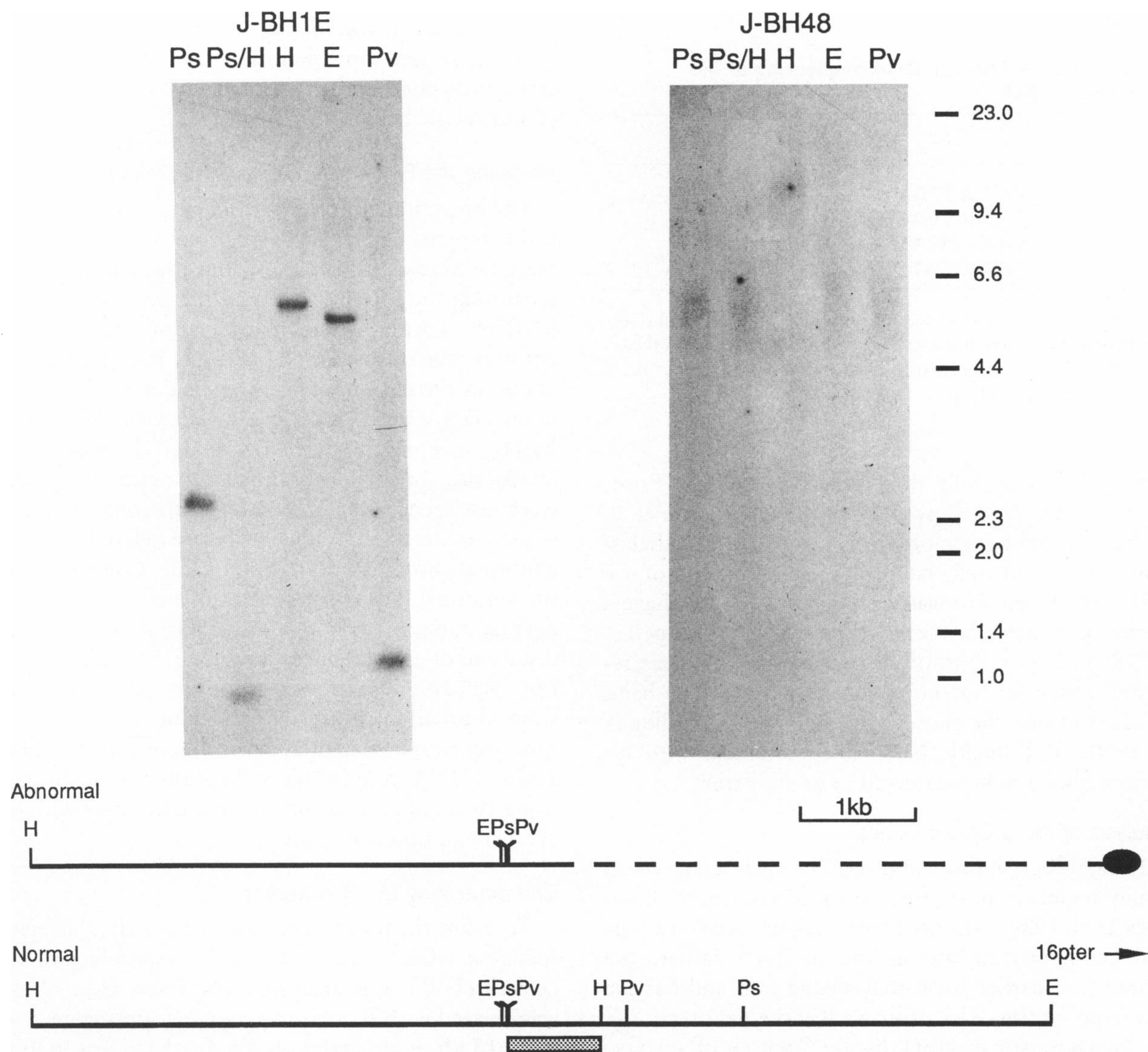

Figure 2 Analysis of human/mouse hybrid cell lines. The probe PsH 0.9 (indicated by a stippled bar in the map) was hybridized to DNA from hybrids containing either the normal chromosome 16 of the proband (J-BH1E) or the abnormal chromosome (J-BH48). The sizes of λ*Hin*dIII and ϕx 174 *Hae*III markers are shown in kilobases. The restriction maps derived from these data are shown diagrammatically beneath the autoradiographs. The dashed line and oval represent the newly formed telomere of the abnormal chromosome. Ps = *Pst*I; H = *Hin*dIII; E = *Eco*RI; and Pv = *Pvu*II.

However, hybridization of the two proximal fragments (4 kb and 13 kb) to PFGE blots revealed abnormal fragments in the patient which were identical in size to those obtained with the probe GGG1 (data not shown). Neither of the more distal fragments (6 kb and 9 kb) detected the abnormal bands. The breakpoint was, therefore, localized to the 13-kb *Bam*HI fragment (*j*, in fig. 1*c*).

Similar analysis using subsegments of the 13-kb *Bam*HI fragment localized the breakpoint within a 6-kb *Hin*dIII fragment (*k*, in fig 1*c*) which was subcloned (plasmid pUW9.1; fig. 1*d*). When *Pst*I-digested subfragments of pUW9.1 were used, dosage analysis indicated that a 0.6-kb *Pst*I fragment (Ps 0.6; *m*, in fig. 1*d*) is present in two copies but that the adjacent 0.9-kb *Pst*I/*Hin*dIII fragment (PsH 0.9; *n*, in fig. 1*d*) is present as only a single copy.

To simplify the mapping, both the normal and ab-

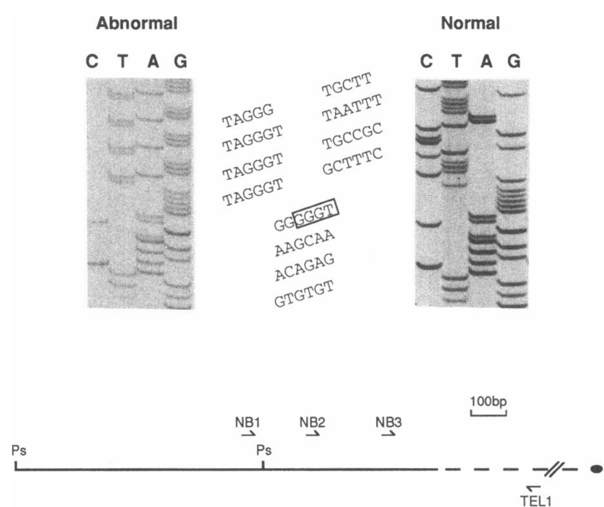

Figure 3 Sequence analysis at the breakpoint. The sequence at the breakpoint is shown, along with the corresponding normal chromosome 16 sequence. The 4-bp motif present in the normal sequence, as well as in the telomere repeat sequence, is boxed. A map of the breakpoint shows the primers used in PCR and sequencing. The dashed line and oval represent the newly formed telomere of the abnormal chromosome. Ps = *Pst*I.

normal chromosome 16 homologues were introduced separately into a MEL cell line. When DNA from a hybrid cell line (J-BH1E) containing the proband's normal chromosome 16 was used, the probe PsH 0.9 detected discrete bands, as expected from the restriction map (fig. 2). When the same probe was used to analyze the hybrid J-BH48, containing the deleted chromosome 16 homologue, no discrete bands were present. However, faint smears of hybridization were seen. Hybridization to *Pst*I, *Eco*RI, and *Pvu*II digests resulted in smears in the size range 5.0–6.5 kb. The proximal restriction site for each of these enzymes lies approximately 0.5 kb from the breakpoint, suggesting that 5–6 kb of variable-length DNA has been added beyond the site of breakage (fig. 2). Consistent with this interpretation, the smear of hybridization in a *Hin*dIII digest was in the size range 10–12 kb, because the restriction site in this case is 5.5 kb proximal to the breakpoint. This length variation could be accounted for by the addition of telomeric sequence heterogeneous in length beyond the site of cleavage.

Sequence Analysis

To determine the precise nature of the deletion, the breakpoint region was amplified by PCR using oligonucleotides which match normal sequences close to the breakpoint, together with an oligonucleotide homologous to the canonical telomere repeat TTAGGG (Wilkie et al. 1990*b;* table 1). After electrophoresis and Southern blotting, hybridization with the probe PsH 0.9 revealed a smear of specific product of size 0.6–6 kb, amplified from genomic DNA samples from the patient and the hybrid cell line J-BH48 (data not shown). No specific hybridization was detected when DNA from either the hybrid J-BH1E or a normal control sample was amplified. Direct sequence analysis showed (*a*) identical breakpoints in the genomic and hybrid DNA and (*b*) that telomeric repeat sequence $(TTAGGG)_n$ (Moyzis et al. 1988; Morin 1989) begins immediately beyond the site of breakage (fig. 3). A 4-bp motif (GGGT) present at the site of telomere repeat addition (fig. 3, *box*) makes exact localization of the chromosome break indeterminate.

Discussion

The identification of a case of ATR-16 with a molecular lesion confined to chromosome 16 confirms that monosomy for 16p13.3 material alone (without aneuploidy for a second chromosome) can lead to mental handicap. This case is potentially valuable for defining the phenotype of the condition. When the data reported here are combined with observations of patients with normal intelligence who have well-defined deletions near the α-globin locus (Higgs et al. 1989; Harris et al. 1990; Villegas et al. 1990; Wilkie et al. 1990*b*), it is possible to identify a minimal region of approximately 1.7 Mb on chromosome 16, within which at least one gene important for mental development must be located.

This region extends from the breakpoint of the abnormal chromosome of this patient (BO) to approximately 95 kb proximal to the α-globin gene complex (fig. 4). How many genes are likely to be located within this 1.7-Mb region? A systematic search for expressed sequences, conducted over 140 kb around the α-globin complex, identified at least seven genes, including those in the complex (Vyas et al. 1992). Moreover, a uniformly high density of "rare-cutter" restriction sites has been reported across the whole 1.7-Mb region (Harris et al. 1990). Such sites are characterized by the presence of CpG dinucleotides, which in turn are usually associated with genes (Lindsay and Bird 1987). If it is assumed that these data are representative of the entire 1.7-Mb region, then this region may contain more than 80 genes. Obviously, if this proves to be the case, those genes which contribute to normal mental development

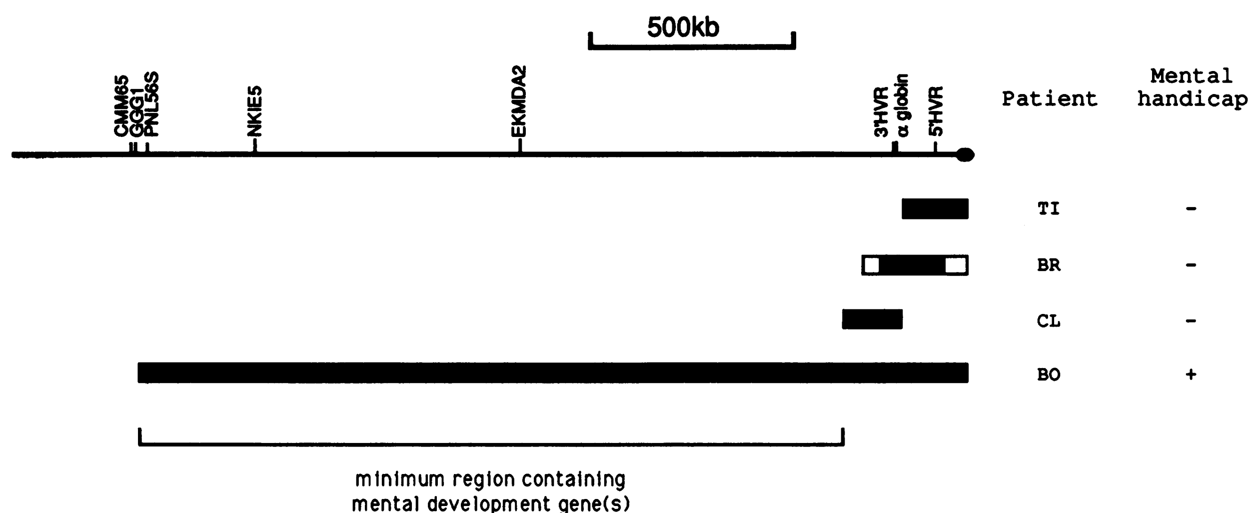

Figure 4 Minimum region of chromosome 16, which is involved in ATR-16. A map of the chromosome is shown above the deletions, which are described elsewhere. (The 16pter polymorphism described elsewhere [Wilkie et al. 1991] has not been shown, for reasons of simplicity.) Blackened bands indicate the known extent of the deletion, and unblackened boxes indicate its maximum length where the breakpoint has not been localized. TI is the mutation reported by Wilkie et al. (1990*b*); a mother and son have a terminal truncation of one homologue of chromosome 16 but are of normal intellect. PFGE analysis suggests that patients with the BR mutation have a deletion of approximately 230 kb from the short arm of chromosome 16 (Harris et al. 1990); they are also intellectually normal. The CL mutation is an interstitial deletion of approximately 120 kb which includes the α-globin genes, but there is no history of mental handicap in this patient (authors' unpublished data). BO is the case reported here.

may be difficult to identify, even if it is assumed that the phenotype of ATR-16 is due to monosomy for one, or a few, critical genes. Alternatively, the phenotype may result from the cumulative effect of the deletion of multiple genes, each making a small, incremental contribution. In this case, patients with small deletions who present with α-thalassemia (for examples, see fig. 4) will be monosomic for relatively few genes, so that the drop in IQ will not be significant, resulting in the false conclusion that hemizygosity for these regions does not contribute to the mental handicap. The resolution of these possibilities will require the analysis of more patients with naturally occurring deletions of chromosome band 16p13.3. A nested set of 16p truncations would be ideal for this purpose.

The data presented here shed further light on the sequence requirements for the in vivo stabilization of broken human chromosomes by the addition of telomere sequence $(TTAGGG)_n$. A 4-bp sequence (GGGT) at the junction site of the abnormal (BO) chromosome corresponds both to normal chromosome 16 sequence and to part of the human telomere hexamer repeat. The only other naturally occurring human chromosome truncation reported is that associated with the $(\alpha\alpha)^{TI}$ chromosome, which has a similarly ambiguous 3-bp sequence (GTT) at the junction site (Wilkie et al. 1990*b*). Recent in vitro studies using human and *Tetrahymena* telomerase to elongate single-stranded primer oligonucleotides have demonstrated that the sequence requirements for chromosome healing may be less stringent than previously thought (Harrington and Greider 1991; Morin 1991). Both studies showed that telomere synthesis can occur when only minimal complementarity to the RNA template of telomerase is present at the 3′ terminus of the oligonucleotide (four G residues in the case of *Tetrahymena;* two to four complementary bases in the case of human telomerase). Although those reports do not explain how telomerase would be able to recognize the double-stranded DNA break associated with chromosome truncation, they do suggest that a short 3′ overhang at the junction site of either the $(\alpha\alpha)^{TI}$ or the abnormal BO chromosome may be sufficient to prime telomere synthesis. It is possible that a 3′ overhang could have been generated during chromosome breakage. Alternatively, it might have resulted from the action of exonuclease at the double-strand break or from DNA replication of the broken chromosome.

A G-rich telomere-like sequence reported 10 bp proximal to the junction site of the $(\alpha\alpha)^{TI}$ chromosome was previously tested for its effect on the efficiency of human telomerase (Morin 1991). This sequence enhanced the ability of telomerase to synthesize repeat

sequences on primers with complementary 3′ termini, but it was not sufficient to overcome poor complementarity at the 3′ end of the oligonucleotide. *Tetrahymena* telomerase will add telomere sequences to primers which are noncomplementary at the 3′ end, provided that at least two telomere repeats are present at the 5′ end of the oligonucleotide (Harrington and Greider 1991). No telomere-like sequence was detected within 500 bp of the de novo truncation site in BO (data not shown), suggesting that a G-rich sequence is not an absolute requirement for chromosome healing in vivo in humans.

Unlike the human chromosome truncation $(\alpha\alpha)^{TI}$ reported elsewhere (Wilkie et al. 1990*b*), the mutation reported here was a de novo event. Sequence analysis of genomic DNA both from peripheral blood mononuclear cells and from EBV-transformed lymphocytes revealed no heterogeneity in the truncation breakpoint. Therefore, there is no evidence for the generation of heterogeneous ends because of either DNA replication or the action of exonucleases in different tissues prior to the addition of telomeric sequence to the broken chromosome. This suggests that repair occurred either in germ-line cells, which are thought to have the highest telomerase activity (Allshire et al. 1988; Cross et al. 1989), or early in embryogenesis. Examination of more tissues will be required to determine the timing of such events.

The molecular mechanism underlying the deletion of chromosome 16 material in patient BO may be relevant to contiguous gene syndromes which have been localized to the terminal region of other chromosomes. For example, terminal deletions of the short arms of chromosomes 4 and 17 are associated with Wolf-Hirschhorn syndrome (Lurie et al. 1980) and Miller-Dieker syndrome (Dobyns et al. 1984, 1991), respectively. Some patients with these syndromes may provide further examples of human chromosome truncations where a normal karyotype shows no evidence of microdeletion or cryptic translocation. We have observed at least three other chromosome 16 truncations which terminate distal to the α-globin genes (authors' unpublished data). The characterization of the breakpoints of these chromosomes may help to clarify the sequence requirements and molecular mechanisms involved in healing chromosome breaks in vivo.

Acknowledgments

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