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Molecular Definition of the Smallest Region of Deletion Overlap in the Wolf-Hirschhorn Syndrome

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Summary

Wolf-Hirschhorn syndrome (WHS), associated with a deletion of chromosome 4p, is characterized by mental and growth retardation and typical facial dysmorphism. A girl with clinical features of WHS was found to carry a subtle deletion of chromosome 4p. Initially suggested by high-resolution chromosome analysis, her deletion was confirmed by fluorescence in situ hybridization (FISH) with cosmid probes, E13 and Y2, of D4S113. To delineate this 4p deletion, we performed a series of FISH and pulsed-field gel electrophoresis analyses by using probes from 4p16.3. A deletion of ~2.5 Mb with the breakpoint at ~80 kb distal to D4S43 was defined in this patient and appears to be the smallest WHS deletion so far identified. To further refine the WHS critical region, we have studied three unrelated patients with presumptive 4p deletions, two resulting from unbalanced segregations of parental chromosomal translocations and one resulting from an apparently de novo unbalanced translocation. Larger deletions were identified in two patients with WHS. One patient who did not clinically present with WHS had a smaller deletion that thus eliminates the distal 100–300 kb from the telomere as being part of the WHS region. This study has localized the WHS region to ~2 Mb between D4S43 and D4S142.

Introduction

Wolf-Hirschhorn syndrome (WHS), characterized by mental and growth retardation, a distinct pattern of dysmorphic features, and a variety of physical abnormalities, is caused by a partial deletion of the short arm of chromosome 4 (4p) (Hirschhorn et al. 1965; Wolf et al. 1965). The size of WHS deletions ranges broadly, from approximately one-half of the short arm to as subtle as being cytogenetically undetectable (Lurie et al. 1980; Wilson et al., 1981; Curry et al. 1982; Preus et al. 1985). Such variations do not result in significant differences in WHS phenotype, suggesting that the relatively few genes responsible for the major characteristics of WHS are clustered in a defined region on 4p. Considerable evidence indicates that the WHS critical region is located within the distal portion of chromosome 4p16. Not only have deletions of distal chromosome 4p been revealed by cytogenetic studies of most WHS patients (Lurie et al. 1980; Wilson et al. 1981; Curry et al. 1982; Preus et al. 1985), but submicroscopic deletions of the distal 4p16 (4p16.3) have also been detected by RFLP analysis of DNA markers from 4p16.3 in some WHS patients with apparently nomal chromosomes 4 (McKeown et al. 1987; Altherr et al. 1991; Anvret et al. 1991). Moreover, there is another clinically recognizable condition associated with an interstitial deletion of 4p that is believed to involve a more proximal region of 4p than that seen in WHS (Francke et al. 1977).

We have studied a child who has clinical features of WHS and a subtle deletion of chromosome 4p (Gandelman et al. 1991). The deletion was suggested by high-resolution chromosome analysis and was subsequently confirmed by fluorescence in situ hybridization (FISH) using probes from 4p16.3. Detailed molecular analysis of a number of DNA markers from 4p16.3, identified in studies to isolate the Huntington disease (HD) gene, revealed a deletion of 2.5 Mb in

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our patient, which appears to be the smallest deletion identified, to date, in WHS patients. To further refine the WHS region, we have also studied three patients with presumed 4p deletions resulting from unbalanced translocations. Two of them, presenting clinically as WHS, were found to carry larger deletions including this 2.5 Mb segment, and the third patient, who did not have WHS, had a much smaller deletion, which thus excludes the distal 100–300 kb from the telomere as being part of WHS region. As a result of the present study, we have refined the WHS region to ~2.2 Mb, distal to D4S43 and proximal to D4S142, within chromosome 4p16.3.

Patients, Material, and Methods

Patients Studied

Four unrelated cases with chromosome 4p deletions were studied. The first patient, CM, with clinical features of WHS, is a Caucasian girl. She was born the 1,960-g product of a full-term, uncomplicated pregnancy to a 29-year-old G_1P_1 mother and a 31-year-old diabetic father. Prenatal history was unremarkable, and the family history is notable only for a maternal aunt with a developmental delay. The child was born by spontaneous vaginal delivery, with Apgar scores of 9 at 1 min and at 5 min. Small size and microcephaly were noted at birth, and she had a poor suck, as well as feeding difficulties, in the neonatal period. At 7 mo developmental delay was documented, and at 1 year she developed a seizure disorder. On examination at 15 mo her weight was 7.7 kg, length was 69.5 cm, and head circumference was 42 cm (all below the fifth percentile). The child had a broad forehead with a prominent glabella and mild facial asymmetry. There was relative hypertelorism, arched eyebrows with medial thinning, and upslanting fissures. The nose was broad with a wide, prominent nasal bridge, and there was a short philtrum with thin lips and down-slanting, carplike mouth. The ears were mildly posteriorly angulated, with minimal lobes. She had hypoplastic nipples, clinodactyly, and small, slender, tapered fingers. Development was delayed, and there was esotropia, poor head control, hypotonia, and increased deep-tendon reflexes. Initial cytogenetic study of her lymphocytes revealed a normal karyotype. High-resolution chromosome analysis suggested a possible subtle deletion of 4p16.3 (fig. 1).

Three cell lines of skin fibroblasts with presumptive 4p deletions as the result of unbalanced translocations were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). Two cases, GM 8210 and GM 1183, were clinically diagnosed as WHS and had a karyotype of 45,XX, – 4, – 21, + t(4;21)(p16.2;q21) (Carpenter et al. 1987) and 46,XY, – 4, + der(4)t(4;12)(p16;p11) pat (Schmutz and Simpson 1983), respectively. The patient GM 8210 was also monosomic for chromosome 21q21→pter and exhibited some features seen in other cases with deletions in proximal

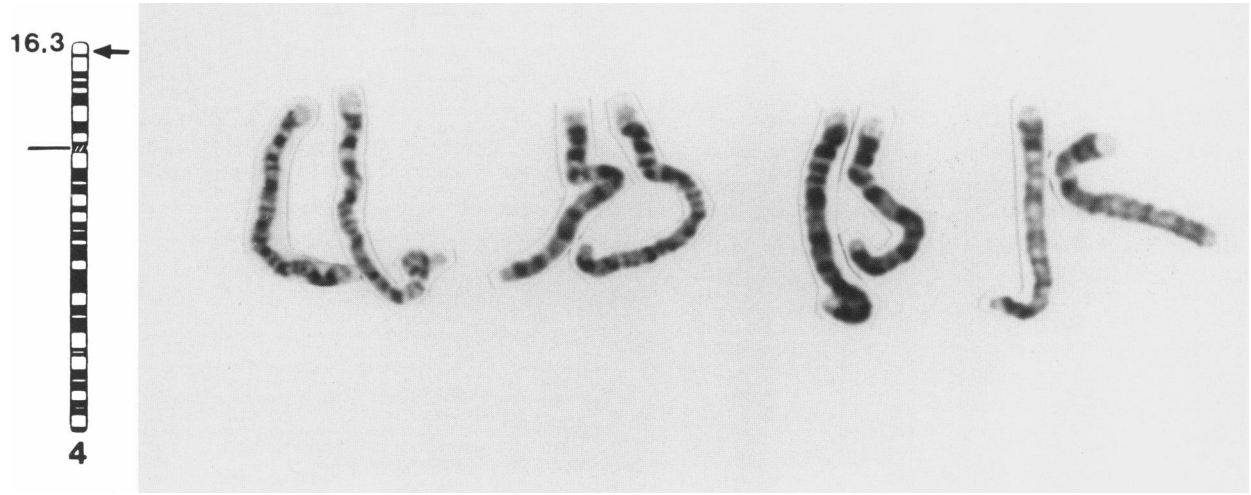

Figure 1 Four pairs of G-banded chromosomes 4 of patient CM. The chromosome with a possible subtle deletion of 4p16.3 is placed at the left of each pair. An ideogram of chromosome 4 at the 850-band stage of resolution (Harnden and Klinger 1985) is shown, with an arrow indicating the deletion breakpoint.

21q. The patient GM 1183 was reported to have WHS, but no detailed description of his clinical features was given. Although GM 1183 carried a duplication of chromosome 12p in addition to a 4p deletion, the predominance of 4p− anomalies is not unexpected, because deletion of genetic material usually has more deleterious effects than does duplication. The third case, GM 1220, with a karyotype of 46,XX, −4, +der(4)t(4;7)(p16;q34) mat, had a deletion of 4p and a duplication of 7q34→qter. He did not present with WHS (Jackson et al. 1977; L. Jackson, personal communication) but showed hypotonia and cardiac defects consistent with the anomalies reported in cases of partial 7q duplication (Bartsch et al. 1990).

DNA Probes

A large number of DNA clones from the region 4p16.3 were generated during the course of studies to identify the HD gene. The cosmid probes used for FISH in this study are listed in table 1. Most of them have been precisely localized on a long-range restriction map of 4p16.3 (Bućan et al. 1990; Whaley et al. 1991). Three probes—1S74, A10, and BJ11—located proximal to 4p16.3 were also employed for FISH (Lin et al. 1991). A chromosome 4 centromere-specific probe p4.3 (obtained from Drs. Baldini and Rocchi, Alghero, Italy) that is also homologous to the chromosome 9 centromere was cohybridized with each 4p probe in FISH experiments, for chromosome 4 identification. Two plasmid probes, C4H and L6H6, from the 4p cosmids C9A and L6, respectively, were used for pulsed-field gel electrophoresis (PFGE) analysis. One chromosome 13 probe, PHUB8 of D13S5, was used as a control for the amount of DNA loading in the PFGE study.

Chromosome Analysis and FISH

High-resolution chromosome analysis was performed on Giemsa-banded chromosomes from amethopterin-synchronized peripheral blood cultures of patient CM. Lymphocytes from CM were transformed by Epstein-Barr virus, and this lymphoblastoid cell line was used for chromosome preparation for FISH and PFGE analyses.

FISH was performed essentially as described by Lichter et al. (1990). The cosmid probes and centromere probe, labeled with biotin-11-dUTP by nick-translation, were used for hybridization at a concentration of 6 μg/ml and 500 ng/ml, respectively. Detection of the biotinylated-probe hybridization sites was achieved using fluorescein isothiocyanate-conjugated avidin (FITC). Chromosomes were counterstained with propidium iodide (PI) and visualized under a photomicroscope equipped with FITC and PI epifluorescence optics. Photographs were taken with Ilford XP1 400 film.

PFGE

Lymphoblastoid cells from patient CM and two normal individuals were used for this study. The methods for DNA preparation in agarose blocks, as well as the restriction digestion of DNA in agarose blocks,

Table 1

Results of FISH Analysis

Probe	Locus[a]	Presence of Hybridization Signal in Cases[b]			
		CM	GM8210	GM1183	GM1220
2R88	D4S142	−	−	ND	−
CD2	D4S90	ND	ND	ND	+
I14	ND	−	−	−	+
A157.1/A157.6	D4S111	−	ND	ND	+
E13/Y2	D4S113	−	ND	ND	+
L6W1/L6	D4S166	−	ND	ND	+
C9A	D4S43	+	−	−	+
1S74	ND	+	−	−	ND
A10	ND	+	+	+	ND
BJ11	ND	+	+	+	+

[a] Listed in order from the most distal (D4S142; 100 kb from telomere) to the most proximal.

[b] A minus sign (−) denotes that hybridization signal was absent from one chromosome 4; and a plus sign (+) denotes that hybridization signal was present on both chromosomes 4. ND = not determined.

have been described elsewhere (Yang-Feng et al. 1990). The electrophoresis was conducted with 1% agarose gel at 200 V in 0.5 × TBE (1 × TBE = 90 mM Tris, 64.6 mM boric acid, 2.5 mM EDTA, pH 8.3) for 24 h at 14°C, with continuously increasing pulse times of 50–100 s in a counter-clamped homogeneous electric field apparatus (Chef II; Bio-Rad). After electrophoresis, the DNA was transferred to Hybond nylon filters as in regular Southern blotting and sequentially hybridized with probes C4H, L6H6, and PHUB8.

Results

FISH

Cytogenetic analysis of lymphocytes from patient CM suggested a subtle deletion of distal 4p16.3 (fig. 1). This deletion was confirmed by FISH using probes of D4S113. A hybridization signal was detected only on one chromosome 4 and was missing from its homologue, indicating the absence of D4S113 sequence. To determine the extent and nature of the CM deletion, a series of FISH analyses using probes spanning 4p15→pter was performed. As summarized in table 1, CM was found to carry a distal 4p deletion with a breakpoint between loci D4S43 (fig. 2*a*) and D4S166 (fig. 2*b*), covering ~2.5 Mb within 4p16.3.

In an attempt to identify the smallest overlapping deletion in WHS patients, skin fibroblasts from two unrelated patients with WHS and from one patient without WHS, all carrying presumptive 4p deletions, were studied. Results of FISH (table 1) revealed that the two WHS patients (GM 8210 and GM 1183) had similar deletions with the breakpoints within the 4p16 (4p16.1→p16.3) proximal region between probes 1S74 and A10, with A10 being proximal to the breakpoints. However, A10 was previously mapped, by somatic cell hybrid analysis, as being distal to the breakpoint of the t(4;21) translocation in GM8210 (MacDonald et al. 1987; Lin et al. 1991). This discrepancy is probably due to the fact that Southern hybridization with a cosmid probe usually requires the suppression, by some form of blocking, of the repeated sequence elements within it and often reduces the hybridization sensitivity.

These two WHS deletions apparently are larger than that identified in patient CM. In the non-WHS individual (GM 1220), of nine probes examined, eight were retained on both normal and derivative chromosomes 4, and only the most distal one of locus D4S142 was deleted from the derivative 4. Since D4S142 is located 100 kb from the 4p telomere and 200 kb distal to D4S90 (Bates et al. 1990), this much smaller terminal deletion identified in the non-WHS individual thus eliminates the distal 100–300 kb from the telomere as being part of the WHS critical region, which is therefore placed between D4S43 and D4S142.

PFGE Analysis

PFGE analysis was employed to delineate and to precisely localize the breakpoint of the CM deletion. FISH study has demonstrated that D4S43 is the most distal locus retained on CM's deleted chromosome 4. As shown in figure 3*a*, when compared with the normal control, an additional smaller hybridizing fragment of ~80 kb was detected in *Not*I and/or *Mlu* digestions of CM's DNA, by probe C4H of D4S43. The *Nru*I fragment detected in the normal control DNA is about the same size (340 kb) as that published by Bućan et al. (1990) but appeared to be smaller in CM's sample, probably because of the variability of DNA mobility often encountered in PFGE analysis. The same PFGE filter was rehybridized with the probe L6H6 of D4S166 and then with the probe PHUB8 of D13S5. The probe L6H6 detected hybridizing fragments of the same size but with much reduced intensity in CM's samples, when compared both with the control probe PHUB8 (data not shown) and with that of the normal control samples, suggesting that the D4S166 sequence was deleted from one of CM's chromosomes 4, which is consistent with the results of FISH. Since C4H and L6H6 probes hybridize to the same *Not*I fragment of ~200 kb (Whaley et al. 1991), PFGE analysis therefore localized the CM deletion breakpoint as being, at most, ~80 kb distal to D4S43 and within the ~120 kb proximal to D4S166.

Discussion

Molecular evaluation of WHS cases with apparently normal chromosomes provides more precise localization of the phenotype on 4p and thus refines the WHS critical region. We have characterized 4p deletions in four individuals with or without WHS. By FISH and PFGE analysis using probes from region 4p16.3, we have identified a deletion with the breakpoint at about 80 kb distal to D4S43 in WHS patient CM. According to the published detailed physical map of 4p16.3, the size of CM's deletion is ~2.5 Mb (Bućan et al. 1990; Bates et al. 1991; Whaley et al. 1991). There are a number of WHS cases in the literature that are re-

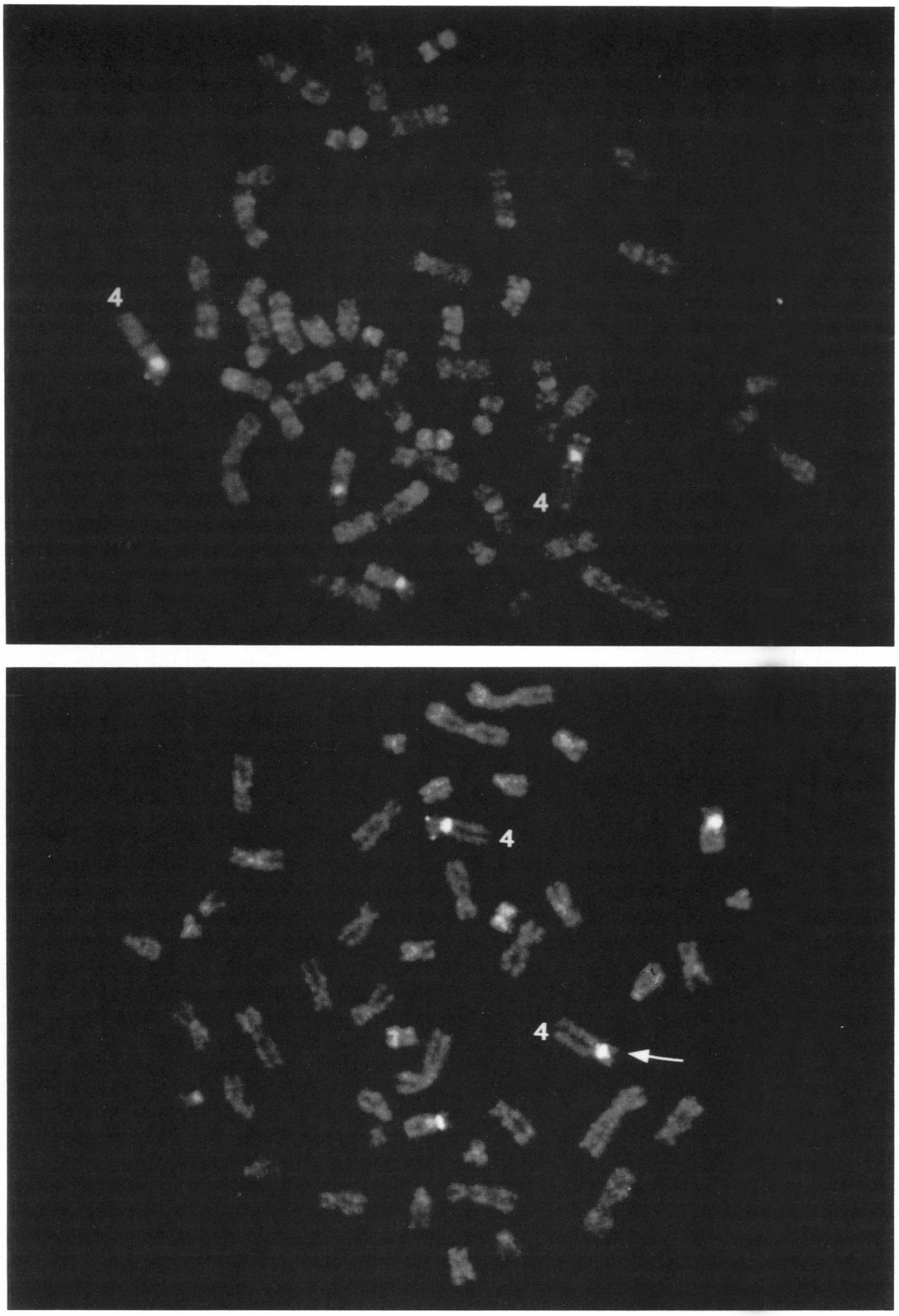

Figure 2 Representative metaphase spreads of CM after in situ hybridization with probes C9A(D4S43) and L6(D4S166) and a centromere-specific probe for chromosomes 4 and 9. Hybridization signal was detected on both chromosomes 4 by C9A (*top*) but only on one chromosome 4 by L6 (*bottom*). The arrow indicates the chromosome 4 lacking the D4S166 sequence.

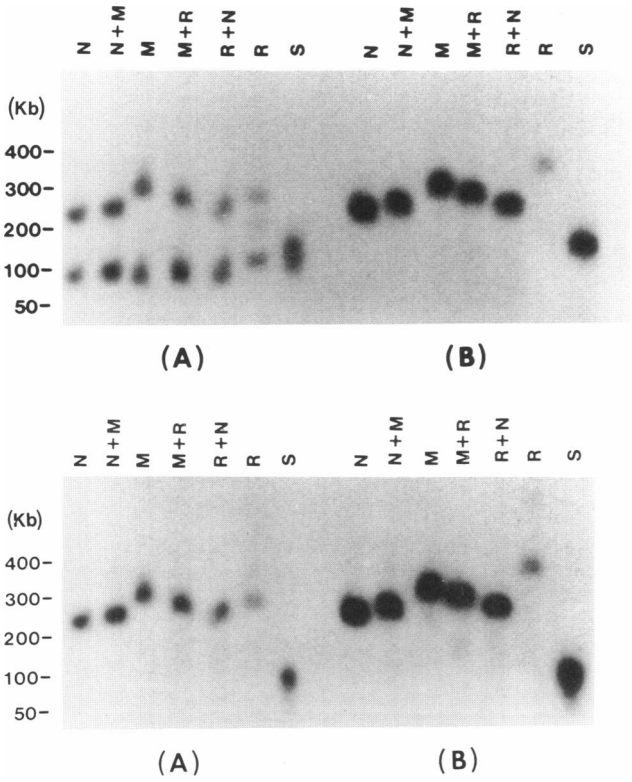

Figure 3 PFGE analysis of DNA from patient CM (A) and normal control (B). Enzymes used for DNA digestion are indicated above each lane. N = *Not*I; M = *Mlu*I; R = *Nru*I; and S = *Sfi*I. *Top,* Additional hybridizing fragment detected by probe C4H (D4S43) in patient CM. *Bottom,* Reduced hybridization intensity revealed by probe L6H6 (D4S166), indicating the deletion of one copy of D4S166 in patient CM.

ported as having submicroscopic 4p deletions. Those cases include simple deletions and subtle translocations of chromosome 4. Most of them carried deletions of 4–5 Mb with breakpoints, either distal or proximal to D4S10, at ~500 kb around D4S10 (Altherr et al. 1991; Curtis et al. 1991; MacLaren et al. 1991; Spiegel et al. 1991). One case had a breakpoint within D4S10 (Anvret et al. 1991), and two cases, with undetermined breakpoint locations in which only two loci were examined, were at least missing sequences of D4S95 (Greenberg et al. 1989) and/or D4S43 (Driscoll et al. 1989). The breakpoints of all these known submicroscopic WHS deletions are apparently proximal to D4S43 (fig. 4). CM's deletion—~2.5 Mb, with a breakpoint at ~80 kb distal to D4S43—is therefore the smallest chromosome 4p deletion that has so far been identified as leading to the WHS phenotype. The 2.5-Mb WHS segment was further narrowed down ~2.2 Mb, since deletion of D4S142 was detected in a non-WHS case (GM 1220) and thus excluded the terminal 100–300 kb (fig. 4).

The proximal ~800 kb of this 2.2-Mb WHS segment overlaps with the HD candidate region, which covers 2.5 Mb spanning D4S10–D4S168 (Bates et al. 1991). It would be expected that some WHS patients may have manifestations of HD, because the HD gene may be deleted in patients with WHS. Such an observation, however, has not been reported, probably because most WHS patients do not reach the age at onset of HD. It is also possible that HD is caused by a mutant gene product, whereas the WHS phenotype is more likely the result of gene dosage effects, with one copy of the relevant gene(s) deleted in WHS patients.

Two expressed genes, α-L-iduronidase (IDUA) (MacDonald et al. 1991) and fibroblast growth factor receptor-3 (FGFR3) (Keegan et al. 1991; Thompson et al. 1991), are located within the 2.2-Mb WHS region. Deficiency of IDUA results in mucopolysaccharidosis type I disorders, including Hurler, Scheie, and Hurler-Scheie syndromes, which are inherited in an autosomal recessive fashion (Neufeld and Muenzer 1989). The map position of IDUA coincides with D4S111 and has placed it outside the HD region (MacDonald et al. 1991). Although one copy of IDUA is deleted in WHS patients, their phenotypes are unlikely to be the result of a reduced amount of IDUA, because of the absence of WHS clinical features in heterozygous parents and in siblings of Hurler patients. The FGFR3 gene, with a high level of expression in brain (Thompson et al. 1991), together with FGF plays a role in both neuronal survival (Anderson et al. 1988; Burgess and Maclag 1989) and differentiation of the central nervous system (CNS) (Murphy et al. 1990) and has been suggested to be an HD candidate gene (Thompson et al. 1991). It may also play a role in WHS, since mental retardation is one of the most consistent features seen in WHS patients and about one-third of the patients were found to have CNS defects at autopsy (Lazjuk et al. 1980). In addition to the CNS defects, the deletion of one copy of FGFR3 may contribute to some other WHS abnormalities, as the FGFR3 transcript is also expressed in kidney, lung, intestine, testes, liver, etc. (Thompson et al. 1991). Furthermore, the expression of FGFR3 in certain tissues, e.g., muscle and spleen, is higher during fetal development than it is in adult baboon and monkeys, a finding compatible with the observation of intrauterine growth retardation in many WHS individuals.

The multiple congenital anomalies of WHS are presumably caused by the effect of several contiguous

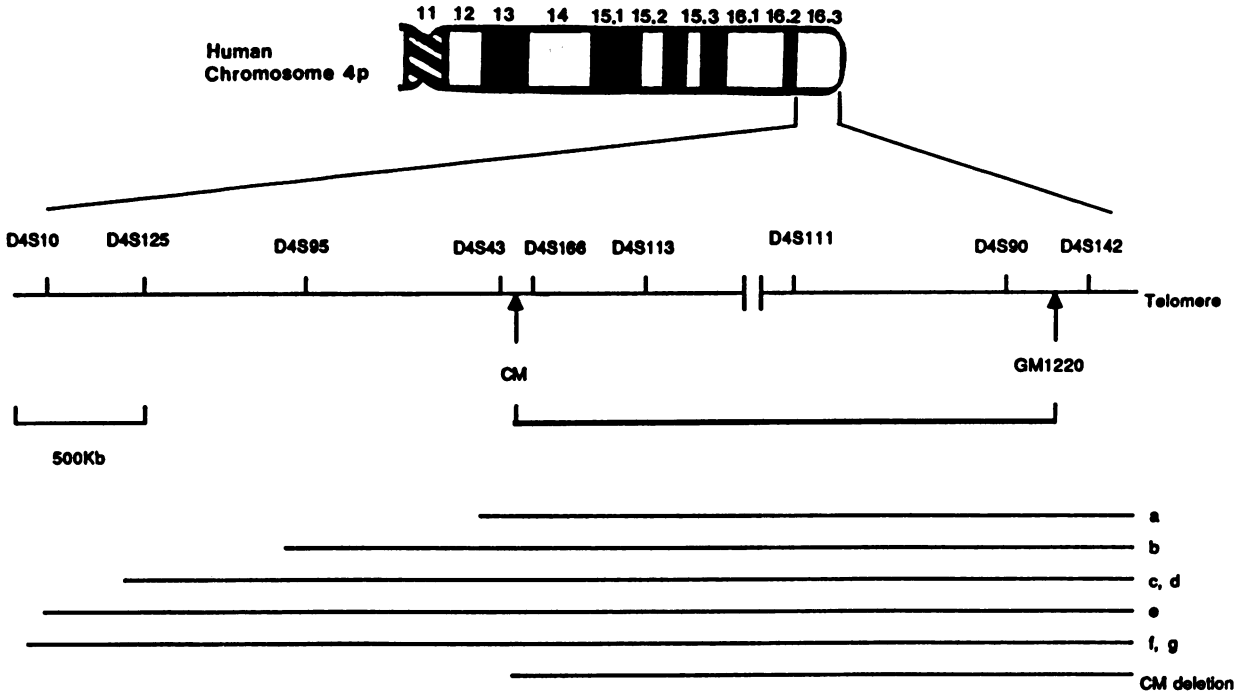

Figure 4 Physical map of chromosome 4p16.3. The gap at ~1.5 Mb from the telomere in the map is indicated (Bućan et al. 1990, Bates et al. 1991). Arrows indicate the breakpoints of CM deletion and GM1220 translocation. The bar below the map illustrates the extent of the WHS region. Published submicroscopic WHS deletions are indicated. a, Driscoll et al. 1989; b, Greenberg et al. 1989; c, Spiegel et al. 1991; d, Curtis et al. 1991; e, Anvret et al. 1991; f, Altherr et al. 1991; and g, MacLaren et al. 1991.

genes deleted from one chromosome 4p. In addition to FRFR3, other expressed sequences in this 2.2-Mb segment, which may also be responsible for WHS-associated abnormalities, have yet to be identified and characterized. The physical map of 4p16.3 is almost complete, and overlapping yeast artificial chromosome clones from distal chromosome 4p have been constructed during the course of studies to isolate the HD gene (Bates et al. 1990). Further molecular characterization of chromosome 4p16.3 will certainly facilitate the identification of the gene(s) responsible for WHS.

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