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Clinical Features and Molecular Analysis of the α Thalassemia/Mental Retardation Syndromes. II. Cases without Detectable Abnormality of the α Globin Complex

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Summary

We have identified five unrelated patients, all of north European origin, who have hemoglobin H (Hb H) disease and profound mental handicap. Surprisingly, detailed molecular analysis of the α globin complex is normal in these subjects. Clinically, they present with a rather uniform constellation of abnormalities, notably severe mental handicap, microcephaly, relative hypertelorism, unusual facies and genital anomalies. Hematologically, their Hb H disease has subtly but distinctly milder properties than the recognized Mendelian forms of the disease. These common features suggest that these five "nondeletion" patients have a similar underlying mutation, quite distinct from the 16p13.3 deletion associated with α thalassemia and mild to moderate mental retardation described in the accompanying paper. We speculate that the locus of this underlying mutation is not closely linked to the α globin complex and may encode a *trans*-acting factor involved in the normal regulation of α globin expression.

Introduction

Since the original description of hemoglobin H (Hb H) disease (a severe form of α thalassemia) and mental retardation in three patients of north European origin (Weatherall et al. 1981), we have identified a further 10 patients in whom mental retardation is associated with some form of α thalassemia. Out of the total of 13 patients, nine have Hb H disease and the remaining four have α thalassemia trait (for a review of the genetics of α thalassemia see Higgs et al. [1989] and the accompanying article of Wilkie et al. [1990]. In every case, the occurrence of α thalassemia determinants in the patient's parents is insufficient to explain the hematologic phenotype of the offspring; therefore, an additional mutation appears to have occurred. We surmised that elucidation of the nature of the α thalassemia mutation(s) in these patients might provide insight into the cause of their associated mental handicap.

In the accompanying paper (Wilkie et al. 1990) we show that eight of the above cases, including all four with α thalassemia trait, are readily explained by extensive deletions including the α globin locus in chromosome band 16p13.3. Depending on whether a $-\alpha$ or αα allele was inherited from the other parent, the resulting genotype is either $--/-\alpha$ (patients with Hb H disease) or $--/\alpha\alpha$ (patients with α thalassemia trait). The mental handicap in these patients (subsequently referred to as the "deletion" cases) may be attributed to the monosomy for chromosome band 16p13.3, as well as other associated chromosomal aneuploidies (Wilkie et al. 1990).

By contrast, preliminary analysis of the remaining five patients (all with Hb H disease), the subject of this

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paper, showed completely normal α globin gene maps (αα/αα; "nondeletion" cases): paradoxically, these patients had much more severe mental handicap than any of the deletion cases. Although homozygosity for certain nondeletion α thalassemia mutations ($\alpha^T\alpha/\alpha^T\alpha$) is known to cause Hb H disease in some populations (Higgs et al. 1983*a*; Pirastu et al. 1984), this could not account for the mental retardation except as a coincidental finding. Instead, it seemed more likely that a new, unusual mechanism was responsible for the Hb H disease.

In this paper we present evidence to support this notion. We show that the clinical features of these patients are remarkably similar, which argues strongly against their mental handicap's being a coincidental finding. The hematologic indices of the Hb H disease are consistent between patients but are subtly different from those found in the Mendelian types, again suggesting a distinctly different molecular basis for this condition.

In an effort to determine the nature of the mutation(s) responsible, we describe a number of molecular strategies we have used to detect possible abnormalities in *cis* to the α globin complex, but all results have been normal. These negative findings, together with other circumstantial evidence, suggest that mutation of a *trans*-acting factor, necessary for the normal regulation of the α globin complex, is more likely to be involved.

Material and Methods

Hematologic Analysis

Blood counts, hemoglobin electrophoresis, and measurement of α/β globin chain synthesis ratios were performed by standard methods (Weatherall and Clegg 1981). Brilliant cresyl blue preparations of blood smears for Hb H inclusions were made as described in Galanello et al. (1984).

Hybrid Cell Lines

The somatic cell hybrid lines MS9B1 and MS9D1 were made by the method of Deisseroth and Hendrick (1978) as described by Zeitlin and Weatherall (1983). They contain, respectively, the paternal and maternal chromosomes 16 from patient SW, in a mouse erythroleukemia cell line (MEL) background. Hybrid 7A1 contains a normal chromosome 16 and has been described elsewhere (Zeitlin and Weatherall 1983).

DNA Probes

The large number of probes recognizing sequences spanning the α globin complex used include α1 globin/HBA1 (Lauer et al. 1980), ψζ1 globin/HBZP (Proudfoot et al. 1982), α globin 3′HVR/D16S85 (Jarman et al. 1986), α globin 5′HVR (Jarman and Higgs 1988), L0 (Fischel-Ghodsian et al. 1988), and L1, L2, RA1.4, RA330, RA1.0, and RA2.2 (Nicholls et al. 1987); note that the full names of this group of anonymous probes should be prefixed with the title α globin. These probes map between 105 kb upstream of the α globin genes (α globin RA2.2) and 10 kb downstream (α globin 3′HVR); see Nicholls et al. (1987). In addition, we used two probes which map more proximally in chromosome band 16p13.3, CMM65/D16S84 (Nakamura et al. 1988) and EKMDA2-I/D16S83 (Wolff et al. 1988). The physical and genetic localization of these probes is described in Germino et al. (1990) and Harris et al. (in press).

DNA Analysis

Genomic DNA was prepared from either venous blood, lymphoblastoid cell lines, or mouse/human hybrid cell lines. Southern blot hybridization was performed as described elsewhere (Wilkie et al. 1990).

DNA analysis using probes to the α globin complex was performed as follows: confirmation of family relationships by DNA fingerprinting with α globin 3′HVR (Wilkie et al. 1990); exclusion of α2 and α1 globin *Nco*I restriction-enzyme defects (Pirastu et al. 1984; Moi et al. 1987); exclusion of *Hph*I restriction-enzyme defect (Orkin et al. 1981); analysis of methylation of α globin genes (Bird et al. 1987); pulsed field gel electrophoresis around the α globin complex (Fischel-Ghodsian et al. 1987*b*).

mRNA Analysis

The relative expression of the α2 and α1 globin mRNAs was determined using the procedure of Orkin and Goff (1981), except that mung bean nuclease was substituted for S1 nuclease.

Cytogenetic Analysis

Methods for obtaining high-resolution G-banded karyotypes and in situ hybridization with α globin probe have been described elsewhere (Lamb et al. 1989). Whole plasmid pDH7 (α1 globin) was labeled with ^{3}HdCTP to a specific activity of 5×10^7 dpm/μg, and slides were hybridized overnight, washed in 0.2 × SSC at 60°C, and left to expose for 18 d at 4°C.

Clinical Features

The clinical features of the five patients, all isolated, unrelated cases, are summarized below. Photographs are shown in figure 1, and some measurements are given in table 1. Hematologic data are detailed in a later section. Unless stated otherwise, parents were of north European ancestry, healthy, and unrelated, and there was no history of miscarriages and no significant family history.

Case SW.—This is patient 3 in the original report of Weatherall et al. (1981). He has an older sister and younger brother, both of whom are healthy and intellectually normal. He was born at term + 9 d after an uneventful pregnancy. Vaginal delivery was precipitate; he was cyanosed, flaccid, and did not cry initially. Apgar score was 6 at 1 min. He responded to oxygen but was transferred to the special care unit and observed for 3 d. Severe hypotonia was apparent early on. At age 3 years he was still unable to stand without support, and he started walking at age 6 years. Hb H disease was diagnosed at age 4 years. He has had occasional grand mal convulsions since the age of 4 years but no other major medical problems. Currently aged 16 years, he attends a residential school. He is able to walk and feed himself but is otherwise totally dependent. He has virtually no comprehension of speech, no intelligible expressive speech, and no bowel or bladder control.

On examination, he is microcephalic with relative hypertelorism (table 1), epicanthic folds, a depressed nasal bridge, a small triangular nose with anteverted nares, crowded teeth, a large, often protruded tongue, and relatively simple ears (figs. 1*A* and 1*B*). In addition, at age 16 years he has short stature, hypotonia with a postural scoliosis and pes planus, an overriding fourth left toe, and a shawl scrotum with a small left testis at the inguinal ring and an impalpable right testis. Dermatoglyphics are normal (no arches). He has a small amount of axillary and pubic hair (Tanner stage 2). His testosterone level at age 16 years was 1.5 nmol/liter (normal postpubertal male range 14–42); luteinizing hormone 5.2 U/liter (normal 3.0–8.0), and follicle-stimulating hormone 9.3 U/liter (normal 0.5–5.0). High-resolution cytogenetic analysis and fragile X screening were normal (46,XY).

Case TH.—This boy has two healthy sisters. His mother, maternal aunt and maternal grandmother are said to have been anemic. He was born 5 d preterm. Variable fetal heart rate and meconium-stained liquor were noted during labor. Delivery was vaginal and he was initially cyanosed. He was observed in an incubator for 24 h and went home at 10 d. Marked hypotonia and a poor suck were noted early on. He had left-side talipes, which responded to splinting, and a squint. Hb H disease was diagnosed at age 21 mo. At age 23 mo he had a fundoplication for severe gastric reflux, which had resulted in gross emaciation. He had a single grand mal seizure at age 3 years and further seizures at age 8 years. A unilateral orchidopexy was performed at age 6 years because of bilateral undescended testes.

Table 1

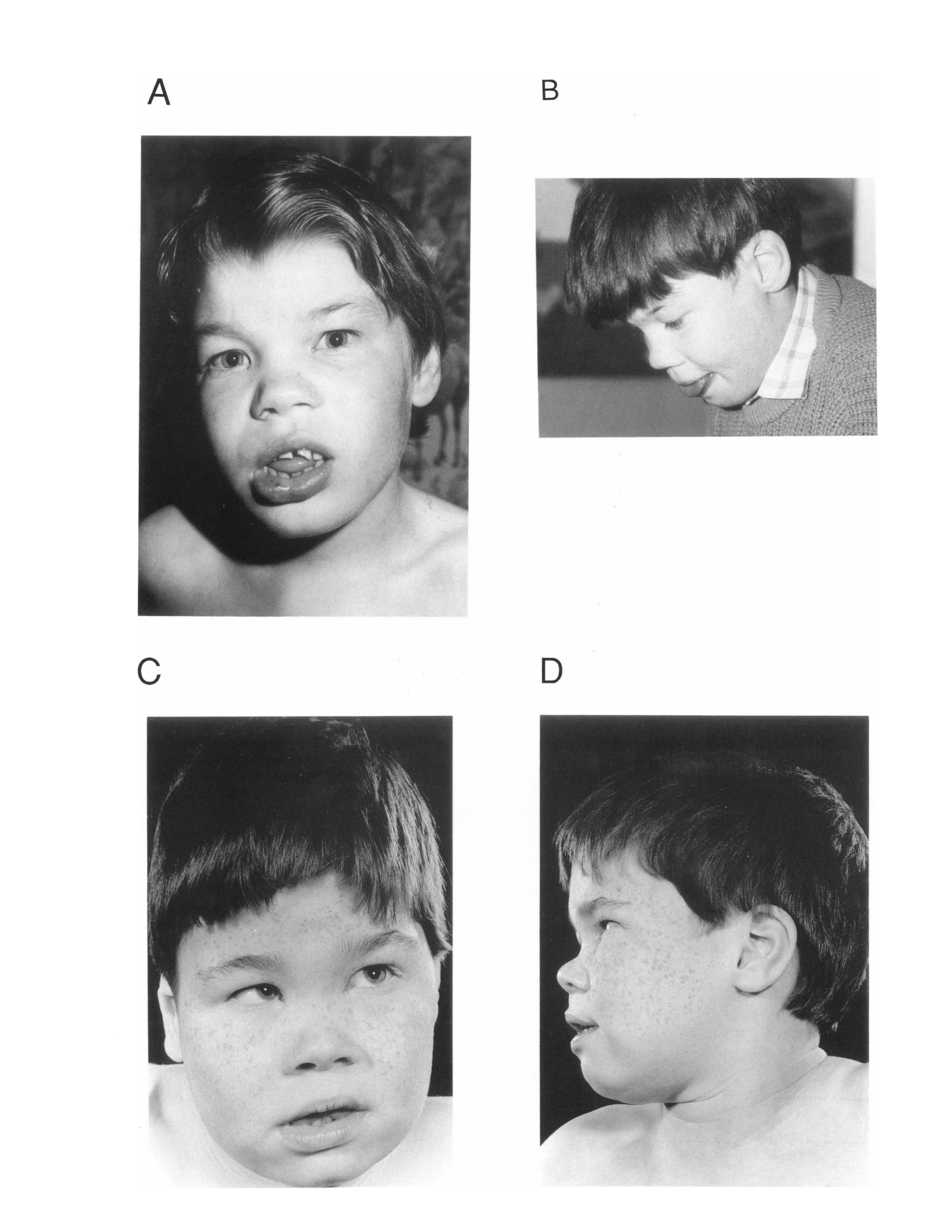
Some Clinical Features of the α Thalassemia/Mental Retardation Patients (Nondeletion Type)

Case	Sex	Age of Parent at Child's Birth (years)		Birth Weight (g)	Head Circumference at Birth (cm)	Age at Exam (years, mo)	Head Circumference (cm)	Inner Canthal Distance (cm)	Height (cm)
		Father	Mother						
SW	M	30	26	3,780 (60)	33.3 (3)	16, 2	50.2 (<<3)	3.2 (65)	136 (<<3)
TH	M	26	24	3,440 (40)	33.6 (10)	9, 2	48.9 (<3)	2.9 (40)	81[a] (<3)
PT	M	33	31	3,900 (75)	34.2 (20)	1, 10	46.5 (5)	ND	ND
NE	F[b]	31	30	2,440 (10)	31.0 (10)	5, 2	46.0 (<<3)	2.6 (25)	107 (40)
PE	M	?	19	2,660 (3)	30.5 (<3)	0, 7	40.7 (<3)	ND	ND

NOTE.—Standard centiles are shown in parentheses. ND = not determined. Single less than and greater than signs (<, >) indicate values 2–3 SD from mean; double signs (<<, >>) indicate values more than 3 SD from mean.

[a] Measured at age 2 years 8 mo.

[b] Female phenotype but XY sex-chromosome constitution.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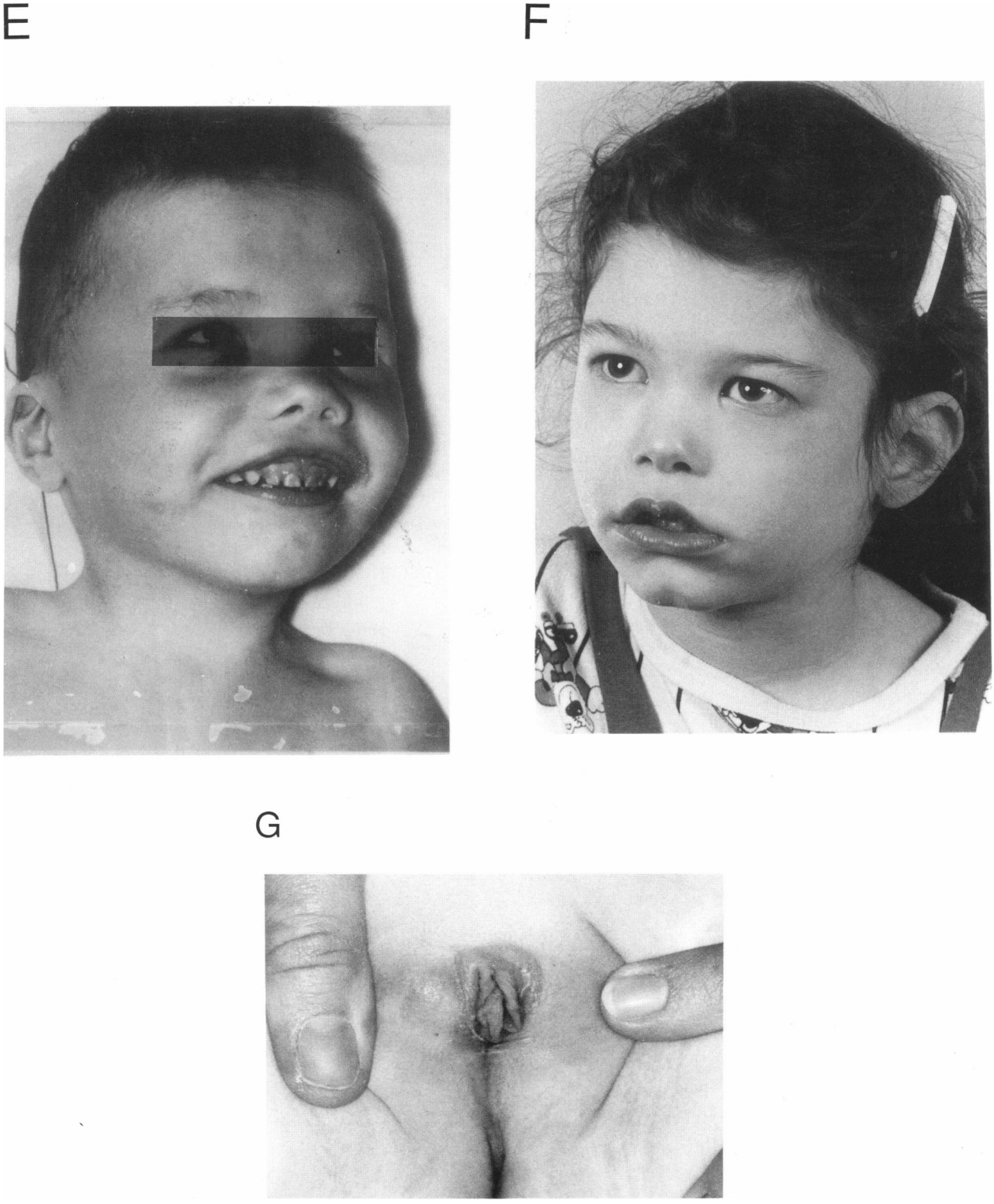

Figure 1 Clinical features of the α thalassemia/mental retardation patients (nondeletion type), *A* and *B*, Case SW aged 12 years (*A*) and 16 years (*B*); *C* and *D*, case TH aged 8 years; *E*, case PT aged 22 months; *F*, facial appearance of case NE aged 5 years; *G*, external genitalia of case NE aged 4 years.

He started sitting at age 4 years and walking with support at age 7 years. At age 8 years he is still unable to express or comprehend any speech.

On examination, he is microcephalic with relative hypertelorism, epicanthus, a squint, a flat face and nasal bridge, a small triangular nose, and anteverted nares (figs. 1*C* and 1*D*). Teeth and ears are normal. General examination at age 2 years 8 mo showed short stature, mild obesity, postural scoliosis, hypotonia, overlapping fourth toes on both feet and a shawl scrotum with impalpable testes. Dermatoglyphics were abnormal: on the hands, 6 of 10 digits carried arch patterns, whereas the scores for his father and mother were 0 and 2, respectively. High-resolution cytogenetic analysis, including in situ hybridization with α globin probe, was normal (46,XY). A CT brain scan showed moderately severe generalized atrophy of the cerebral hemispheres but no other abnormality.

Case PT.—This is case 2 of Weatherall et al. (1981) and had previously been reported as a single case (Sjolin et al. 1964). He has no siblings. Details of the birth history are given in the original report. Abnormalities noted at birth included low set, deformed ears, postnuchal skin folds, undescended testes, and a hypoplastic scrotum. Hb H disease was diagnosed shortly after birth (the baby had 19% Hb Bart's). Poor suck and severe hypotonia were noted early on. Seizures developed from the age of 13 years. At age 19 years he remained totally dependent, adopting a fetal position and being unable to crawl, feed himself, communicate, or control his urine or feces. At this age he was noted to have microcephaly, relative hypertelorism, a flat face and nasal bridge, and a small broad nose (fig. 1*E*, age 22 mo). General examination revealed symmetric hypertonia. A CT brain scan and cytogenetic analysis (46,XY) were normal.

Case NE.—This phenotypic female has three healthy sisters. Her mother is of Hungarian Jewish origin, and had had one previous spontaneous abortion at 7 wk gestation. Delivery was vaginal at approximately 37 wk gestation (dates uncertain); the baby's condition was good at birth. At age 1 day she was transferred to the neonatal unit because of an unusual facial and genital appearance and poor feeding. Hb H disease was diagnosed at this stage (14% Hb Bart's). High-resolution chromosome analysis revealed an apparently normal *male* karyotype (46,XY).

Examination of her genitalia at this stage showed that she had posterior labioscrotal fusion, absent labia minora, normal labia majora, slight clitoral enlargement with a small amount of erectile tissue, and no palpable gonads. She had a short urethra with a somewhat posterior orifice in a very short vagina. There was no evidence of utriclar or uterine structures when examined by voiding cystourethrogram or pelvic ultrasound at the age of 1 mo. Plasma electrolytes and 17-hydroxyprogesterone were normal, but the testosterone level measured at the age of 6 mo was very low (0.14 nmol/liter), with a very subnormal increment to 0.46 nmol/liter after three daily injections of 1,000 units of human chorionic gonadotropin (a normal response would be an increment to >2 nmol/liter). There was no evidence of a specific defect in the biosynthetic pathway of testosterone or dihydrotestosterone, and her phenotype of male pseudohermaphroditism was presumed to result from severe gonadal dysgenesis of unknown cause.

She continued to be hypotonic and was slow to gain weight. Gastroesophageal reflux was diagnosed at age 1 mo, but this settled gradually. At age 51 mo her motor and language abilities matched the 9-mo level. She was cooing and responding to forceful commands, but had no intelligible speech; she could sit unsupported and crawl but not bear weight. She was unable to use a spoon. Horizontal nystagmus and visual inattention were present, and her visual evoked responses were prolonged bilaterally. She had chronic constipation. She was microcephalic, with relative hypertelorism, epicanthic folds, a flat face and nasal bridge, a small triangular nose, an inverted V-shaped upper lip, and posteriorly rotated ears (fig. 1*F*). General examination showed hypotonia, slight brachydactyly, especially of the fifth fingers, normal fundi, and genital anomalies as described above (fig. 1*G*). Her bone age was 42 mo at a chronological age of 62 mo. CT brain scan and EEG were normal at the age of 8 mo.

At age 5 years she had an exploratory laparotomy because of the risk of neoplasia in the dysgenetic testes. Both testes were extremely small (<5 mm) and each was present in the inguinal canal in association with a hernial sac and a normal-appearing spermatic cord. There was no evidence of any female internal genitalia or other gonadal structures. Cystoscopy revealed a small blind-ending vagina with no cervix. Histological examination of the testes showed that both consisted largely of loose interstitial stroma, with only a few scattered spermatic tubules without lumina. The tubules of the right testis contained only Sertoli cells, but a few spermatogonia remained in the left testis. The ductus deferens and epididymis appeared normal.

Case PE.—This boy is not very well documented clinically, although hematologically the diagnosis is not in

doubt. He has no siblings and was born at term. Initial condition was good, but he was hypotonic and required tube feeding. Examination showed small ears, bilaterally undescended testes, kyphosis with a T5 hemivertebra, and missing left rib at that level. Subsequent development was delayed; at age 16 mo he could just sit with support and his developmental age was 6 mo by Denver assessment. A convergent squint and hypoplastic teeth were noted, but no comment was made on his facial appearance and no photograph is available. Subsequently he was lost to follow-up. No chromosome analysis is available.

Hematologic Analysis

The results of hematologic analysis on the families are presented in table 2. In all cases the presence of Hb H disease in the proband was demonstrated by finding Hb H on electrophoresis and numerous Hb H inclusions on a blood smear. Hb H levels, varying from 0.7% to 6.7%, tended to be rather lower than those found in the Mendelian forms of Hb H disease (expected values vary widely, depending on genotype, from 0.8% to 40%; Higgs et al. 1989). This observation prompted us to examine the other hematologic indices, and figure 2 shows a plot of all available mean cell Hb (MCH) measurements from the probands against age, compared with those observed in the $--/-\alpha$, $--/\alpha^T\alpha$, and $\alpha^T\alpha/\alpha^T\alpha$ genotypes (A.O.M.W. and D.R.H., unpublished results). It can be seen that the degree of hypochromia is remarkably similar between the patients, but is consistently milder than that found in the various types of Mendelian Hb H disease. Therefore, these patients do not conform to any of the currently known Mendelian forms of α thalassemia, rais-

Table 2

Hematologic Analysis of the Families

Family Member	Age (years)	Hb (g/dl)	Mean Cell Volume (fl)	MCH (pg)	Hb H (%)	Hb H Inclusions	α/β Globin Chain Synthesis Ratio	α/β mRNA Ratio
SW (male)	8	10.8	67.8	21.6	2.5	+ + +	.32, .48	0.5
Mother	34	14.3	88.7	29.0	0	–	.85, 1.02	1.8
Father	38	15.4	90.6	30.6	0	–	1.11, 1.44	2.2
Sister	10	11.7	79.0	26.8	0	+	1.02, 1.29	2.4
Brother	4	12.7	80.9	27.2	0	–	.92, 1.3	2.3
TH (male)	2.7	10.8	74.3	23.8	1	+ + +	.39, .48	ND
Mother	26	11.8	88.4	30.1	0	–	1.14, 1.22	ND
Father	28	14.6	87.8	29.7	0	–	.89, 1.06	ND
Sister 1	4.8	11.1	81.7	26.8	0	–	ND	ND
PT (male)	17	10.5	90	25	6.7	+ + +	ND	.4
Mother	48	12.6	113	36	0	–	ND	1.7
Father	50	15.3	109	36	0	–	ND	2.3
NE (female)	4.5	11.7	69.7	21.7	0.7	+ + +	ND	ND
Mother	34	14.0	87.7	29.7	0	–	ND	ND
Father	35	15.6	86.3	29.5	0	–	ND	ND
Sister 1	8	14.1	81.7	27.4	0	–	ND	ND
Sister 2	.9	12.0	81.2	27.6	ND	–	ND	ND
Sister 3[a]	.9	10.7	75.5	24.8	ND	–	ND	ND
PE (male)	2	11.3	74	22.9	3.5	+ + +	0.64	ND
Mother	21	13.5	86	29.9	0	–	1.30[b]	ND
Father	?	16.0	90	32.0	0	–	1.22	ND

NOTE.—The data for the SW and PT families is modified from Weatherall et al. (1981). Duplicate values for the α/β globin chain synthesis ratio are given for the SW and TH families. Normal ranges for adults (±2 SD) are as follows: Hb, males 13.5–17.5 g/dl, females 12–16 g/dl; mean cell volume, 80–100 fl; MCH, 26–34 pg (Dallman 1977); α/β globin chain synthesis ratio, .84–1.28; α/β mRNA ratio, 2.0–2.5. Values found in different α thalassemia genotypes are tabulated by Higgs et al. (1989). The average figures for Hb, mean cell volume, and MCH are 5%–10% lower during childhood, depending on age (see fig. 2). Other hemoglobinopathies were excluded (data not shown). + + + = Hb H inclusions present in >5% of red blood cells; + = inclusions in <0.1% of cells; – = no inclusions seen; ND = not determined.

[a] Small clot in sample.

[b] High background counts.

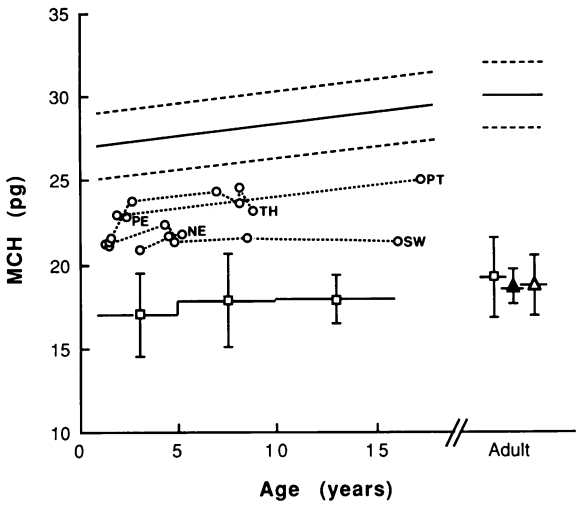

Figure 2 MCH measurements plotted against age. Serial values for the mentally retarded nondeletion patients (O) are joined by dotted lines. These are compared with the normal mean (———) and ± 1 SD (---) lines, and the mean ± 1 SD in the $--/-\alpha$ (—□—), $--/\alpha^T\alpha$ (△), and $\alpha^T\alpha/\alpha^T\alpha$ (▲) types of Mendelian Hb H disease. Normal data are from Dallman (1977) and our unpublished work; the Hb H disease data are updated from Higgs et al. (1989). Insufficient values are available to show the variation of MCH with age in the $--/\alpha^T\alpha$ and $\alpha^T\alpha/\alpha^T\alpha$ genotypes, but these are likely to reflect the trends shown in the normal and $--/-\alpha$ genotypes.

ing the possibility that some different mechanism might be responsible for their Hb H disease.

We next wished wished to examine the evidence for the presence of α thalassemia in the parents and siblings of the probands. Unfortunately, the diagnosis of mild forms of α thalassemia is not straightforward. The most specific of the routinely measured hematologic parameters is the MCH, which declines with increasing α/β globin chain imbalance (Higgs et al. 1989). However, the MCH cannot distinguish the various α thalassemia genotypes in a clear-cut fashion; in particular, there is some overlap between the values of mildly affected individuals and the lower end of the normal range (quoted in table 2): the MCH value (in pg ± 1 SD) found in a sample of 162 adults heterozygous for the single gene deletion genotype ($-\alpha/\alpha\alpha$) was 26.2 ± 2.4, and for 40 adults heterozygous for the nondeletion genotype ($\alpha^T\alpha/\alpha\alpha$) it was 24.6 ± 1.7 (Higgs et al. 1989, and our unpublished data: note that the nondeletion heterozygotes have more severe hypochromia). A further method for corroborating the hematologic findings is measurement of the α/β globin chain synthesis ratio, but this also does not give perfect resolution between genotypes (Higgs et al. 1989). One or both of these approaches have been used to study the previously unpublished families, and we can therefore assign the α thalassemia phenotypes of the various family members with varying degrees of certainty.

Considering first the parents of probands TH, NE, and PE, their MCH values range from 29.5 to 32.0 (table 2). These data alone make it unlikely that any of them possess the common nondeletion α thalassemia genotypes associated with mutation of the α2 globin gene ($\alpha^T\alpha/\alpha\alpha$): the highest MCH in our series of 40 subjects with this genotype was 28.5. We could not on this basis exclude with certainty a nondeletion mutation of the α1 globin gene ($\alpha\alpha^T/\alpha\alpha$), which is likely to be hematologically milder than the $\alpha^T\alpha/\alpha\alpha$ mutation, although only one example of such a mutation has been documented (Moi et al. 1987) and MCH values of 24.4 and 28.0 were recorded in the two affected heterozygotes. For two of the families additional evidence from α/β globin chain synthesis confirmed hematologic normality in the mother of patient TH and both parents of patient PE; the results for the father of patient TH were equivocal (table 2). None of the siblings of any of the three probands had evidence of α thalassemia.

Comparing these findings with those previously published for the SW and PT families (Weatherall et al. 1981; see table 2), quite convincing evidence (observation of reduced α/β globin chain synthesis and/or mRNA ratios) was obtained in both cases, indicating that the mother had a mild α thalassemia defect. Interestingly, both had MCH values (29 and 36 pg) above the range for the ($\alpha^T\alpha/\alpha\alpha$) genotype, suggesting an unusual genetic basis for their abnormality. An important and intriguing finding which was not described in the original paper of Weatherall et al. (1981) is that the sister of patient SW, who has no α/β globin imbalance as measured by either globin chain synthesis or mRNA ratios, nevertheless has had occasional Hb H inclusions observed over several years. To our knowledge, this observation is unique.

In summary, these five patients have an unusual form of Hb H disease with milder hematologic indices than expected. None of them has required any specific treatment for their hematologic abnormality. We can only find definite evidence of α thalassemia in two of their parents, whereas in Mendelian Hb H disease one would expect abnormalities in all 10 of them. Furthermore, the MCH in the two parents in whom α thalassemia is suspected is higher than that found in carriers of the usual nondeletion defects. A daughter of one of these parents consistently has Hb H inclusions, despite a normal α globin synthetic profile.

Molecular Studies

Initial mapping of the α globin genes had shown that both alleles were present in all five probands and that no gross structural abnormalities of the α globin complexes could be detected (Weatherall et al. 1981; and data not shown). This surprising observation clearly distinguishes these cases from the deletion type of α thalassemia/mental retardation syndrome, described in the accompanying article (Wilkie et al. 1990). In designing a rational approach to the further molecular study of the nondeletion patients, two points seemed particularly pertinent. First, since the occurrence of Hb H disease requires a >50% reduction in α globin synthesis (because the $--/\alpha\alpha$ genotype does not cause Hb H disease), both of the allelic α globin complexes must have reduced synthetic output in the probands. Second, a child homozygous for the common Southeast Asian deletion has been reported who, despite complete deletion of the α globin genes (genotype $--^{SEA}/--^{SEA}$), is kept alive by regular blood transfusion: since she appears to be developmentally normal (Bianchi et al. 1986; Fischel-Ghodsian et al. 1987*a*), at least one of the mutation(s) responsible for the nondeletion cases must lie outside the immediate vicinity of the α globin genes. Two models appeared compatible with these requirements (see fig. 3): one α globin allele might be inactivated by a mutation in *cis*, also responsible for the mental handicap, while the other allele carried a conventional nondeletion α thalassemia determinant which we had (in some cases) failed to detect in the parent from whom it had been inherited ("*cis*-inactivation" model); alternatively, a single mutation of an unlinked *trans*-acting factor necessary for the normal expression of the α globin genes could result in the reduced expression of both alleles ("*trans*-inactivation" model). In neither case would

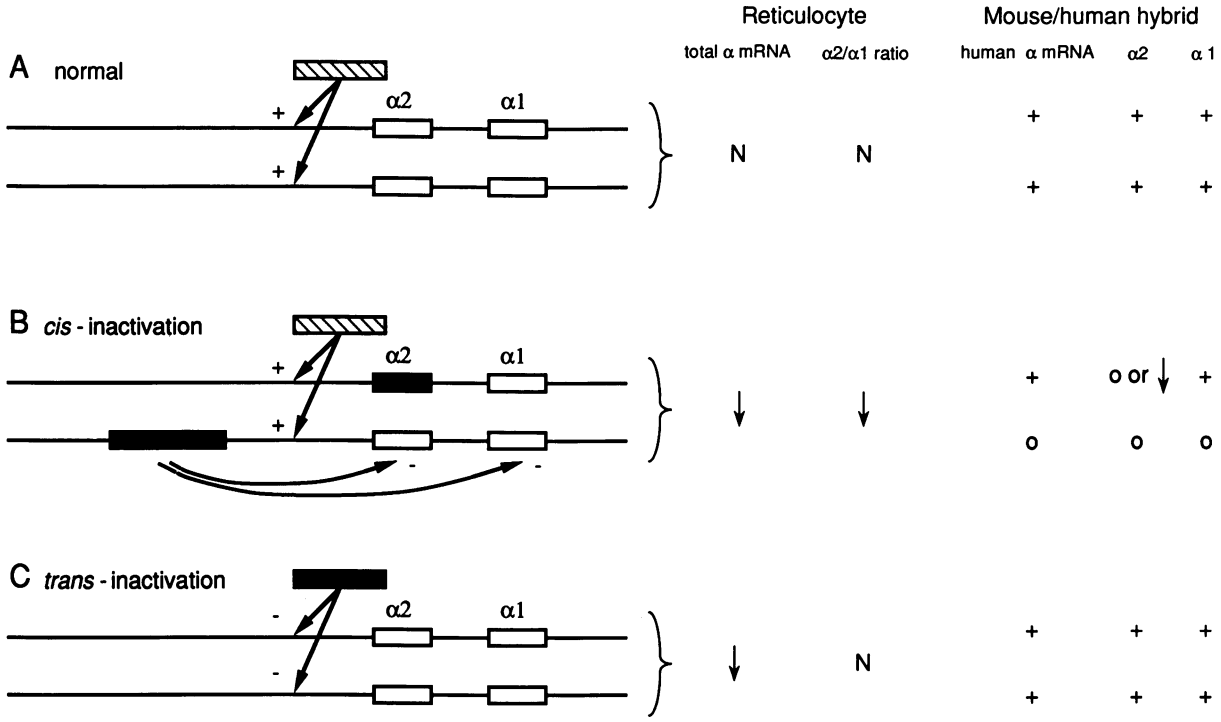

Figure 3 Two possible models to explain the mutation(s) in the nondeletion cases. *Left,* Schematic diagram of the two models (*B, cis*-inactivation; *C, trans*-inactivation), compared with the normal situation (*A*). Functional α globin genes are shown as unfilled boxes; a putative *trans*-acting factor which switches on α globin expression is hatched; mutations leading to loss of function are shown in black (mutation of the α2 globin gene has been arbitrarily chosen for the *cis*-inactivation model). *Right,* Predicted consequences of the two models for α globin mRNA expression, shown for reticulocytes and for the individual chromosomes 16 isolated in mouse/human hybrids. Because a normal human chromosome 16 can synthesize α globin in the hybrid system, the MEL cell is presumed to supply any *trans*-acting factors necessary for α globin expression, and can therefore complement a *trans*-acting mutation. This assay system can reliably distinguish functional from completely nonfunctional α globin alleles, but is less useful for quantitating a modest reduction in function. N = normal level; ↓ = reduced level; + = present; o = absent.

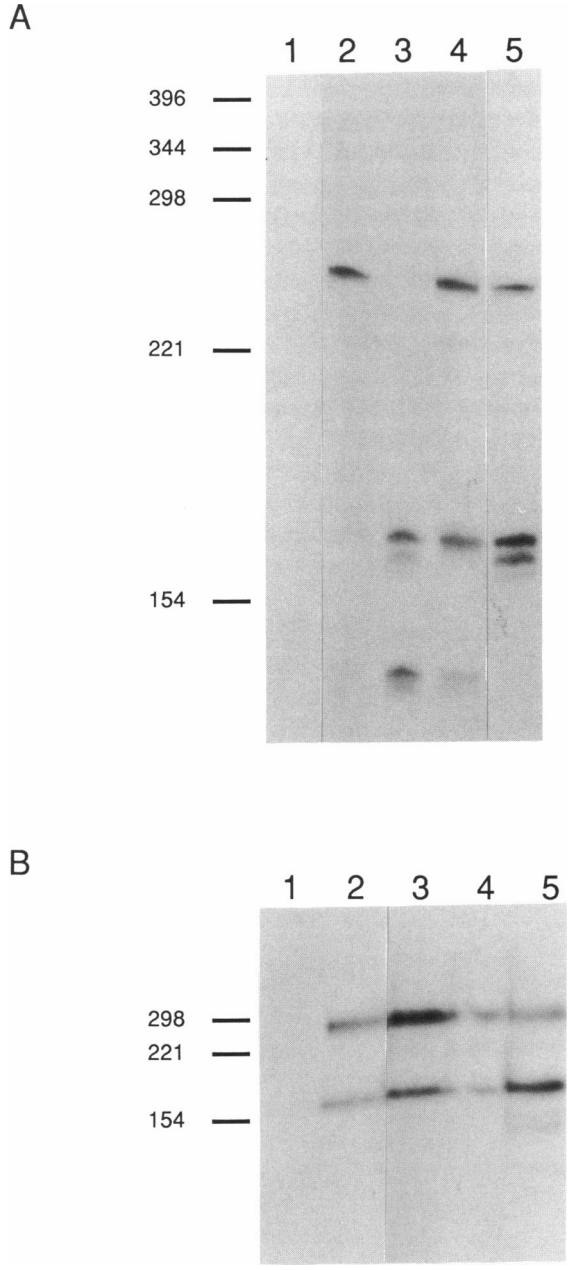

Figure 4 Single-stranded nuclease protection analysis of α globin mRNA. The major protected fragments are ~270 nucleotides (α1-specific) and ~175 nucleotides (α2-specific), with the ~140-nucleotide band representing a partially degraded product. pBR322 DNA digested with *Hin*fI was used as a marker, and sizes are indicated in nucleotides. *A*, mRNA from peripheral blood reticulocyte samples. Lane 1, hydropic infant with −−/−− genotype (no α globin mRNA); lane 2, patient with $-(\alpha)^{20.5}/\alpha^{Hphl}\alpha$ genotype (predominantly α1 mRNA); lane 3, $-\alpha^{3.7III}/-\alpha^{3.7III}$ genotype (α2 mRNA only); lane 4, patient SW; lane 5, ααα/αα genotype (α2>α1 mRNA). *B*, mRNA from mouse/human chromosome 16 hybrids. Lane 1, mouse cell line (585); lane 2, hybrid containing paternal chromosome 16 from patient SW (MS9 B1); lane 3, hybrid containing maternal chromosome 16 from patient SW (MS9D1); lane 4, normal chromosome 16 hybrid (7A1); lane 5, normal reticulocyte RNA. Note that the α1:α2 mRNA ratio appears more balanced in the hybrid samples than in reticulocytes. This phenomenon is unexplained, but has been observed previously in hybrid cell lines containing a normal human chromosome 16 (Zeitlin and Weatherall 1983).

sequencing of the α globin genes elucidate the mutation causing the mental handicap.

One way to distinguish these two models might be to analyse the α globin mRNA for evidence of differential transcription from the α1 and α2 genes. Because many of the common types of Mendelian nondeletion α thalassemia mutations decrease either the transcription of one of the two α globin genes or the stability of its mature mRNA, either α1 or α2 mRNA is often severely reduced or absent (reviewed in Higgs et al. [1989]). Such a pattern of expression from the synthetically active chromosome might be anticipated if the *cis*-inactivation hypothesis were correct (fig. 3*B*). On the other hand, the *trans*-activation mechanism would be consistent with the expression of both α1 and α2 mRNA, but at a reduced level: such a pattern is much less commonly observed in Mendelian nondeletion defects (Liebhaber et al. 1987). Using the method of Orkin and Goff (1981), we analyzed α globin mRNA in patients SW, PT, and PE. Although the total mRNA level was markedly reduced (Weatherall et al. 1981; see table 2), both α1 and α2 species were present (fig. 4*A*, patient SW).

A second prediction of the *cis*-inactivation mechanism is that the two α globin alleles should show markedly disproportionate synthetic function (fig. 3*B*). To test this we isolated hybrid lines in MEL cells containing solely the maternal or paternal chromosome 16 from patient SW. Such cell lines are capable of synthesizing human as well as mouse α globin if the human α globin genes are structurally intact (Deisseroth and Hendrick 1978). Both maternal and paternal SW hybrids were able to synthesize human α globin, excluding the possibility that either chromosome is completely inactivated (data not shown); furthermore, both hybrids expressed α1 and α2 mRNA in a manner indistinguishable from that of a hybrid containing a normal human chromosome 16 (fig. 4*B*), indicating that both α globin genes on each chromosome are transcriptionally active.

Although these results support a *trans*-inactivation mechanism, the methods used would not have been sensitive enough to detect modest discrepancies of synthetic function within or between chromosomes. Therefore we have also searched intensively for direct evidence

Table 3

Summary of Strategies Used to Detect Possible Abnormalities in the Vicinity of the α Globin Complex in the Nondeletion Patients

Type of Abnormality Excluded	Patients Examined
*Nco*I restriction enzyme defect in α2 and α1 genes	SW(g), PT, TH, NE, PE
*Hph*I restriction enzyme defect in α2 gene	SW(g), TH, NE
Altered methylation of α2 and α1 genes in venous blood	SW(g), TH
Altered methylation of α2 and α1 genes in Epstein-Barr virus line	SW(g), NE
Abnormal overlapping restriction enzyme fragments in 120 kb around α globin complex	SW(h), PT, TH, NE, PE
Abnormal fragments on pulsed field digests of *Not*I, *Sal*I, *Nru*I, and *Mlu*I hybridized with α globin probes	SW(h), TH
Hemizygosity for probes CMM65 and EKMDA2-1	SW(h), TH, NE

NOTE.—For patient SW, the abbreviations indicate whether the abnormality was excluded in genomic DNA (g) or the maternal and paternal hybrid cell lines (h).

of molecular abnormalities in *cis* to the α globin complex. We have used a variety of approaches, including examination for known α thalassemia mutations, investigation of DNA methylation patterns, extensive mapping of overlapping restriction fragments, pulsed field gel electrophoresis, in situ hybridization, and the use of linked chromosome band 16p13.3 probes. The results are summarized in table 3: complete data for patient SW and the corresponding hybrid cell lines, and less comprehensive analysis of genomic DNA of the other patients, has not shown any significant abnormality using any of these methods.

In summary, we have been unable to demonstrate defects in *cis* to either allelic α globin complex in any of our patients. Both allelic gene complexes appear to be functional, albeit at a reduced level, and both α1 and α2 genes contribute to this function. These essentially negative findings are consistent with the mutation's being at a locus unlinked to α globin, presumably for a factor which affects α globin expression in *trans*.

Discussion

When the syndrome of Hb H disease and mental retardation was first described (Weatherall et al. 1981), it remained a possibility that the concurrence of these two abnormalities might just be a coincidence. In the accompanying paper (Wilkie et al. 1990), we show that in some patients this is clearly not the case, for we can demonstrate a causative deletion. However, the five nondeletion patients pose more of a problem because, to date, no underlying molecular defect has been found. What is the evidence that this latter group of patients do indeed have a syndrome in common?

First, given that they were originally ascertained hematologically, the similarity in their clinical features is remarkable. The facial features which these children share include microcephaly, marked hypertelorism, epicanthus, a small triangular upturned nose with marked hypoplasia of the nasal bridge, and a flat face. Although the component parts of this facial dysmorphism are rather nonspecific, the final appearance is strikingly uniform (fig. 1). Two other particularly noteworthy characteristics are the genital anomalies (manifesting in one case as male pseudohermaphroditism) and severe mental handicap (IQ <<50). Such congruence of clinical features certainly supports the existence of a unifying cause, and contrasts with the milder mental handicap (IQ 48–76) and much more inconsistent spectrum of abnormalities encountered in the deletion cases (Wilkie et al. 1990).

Second, the Hb H disease in the five nondeletion cases has some unusual but consistent characteristics. The MCH and α/β chain synthesis ratios tend to be higher, and the Hb H level lower, than has been found in either the usual inherited forms of Hb H disease (Higgs et al. 1989) or the four mentally retarded deletion cases who presented with Hb H disease (Wilkie et al. 1990). Furthermore, it is difficult to account for these relatively milder hematologic features in terms of the currently understood genetic mechanisms of α thalassemia. It is also striking that none of the parents has MCH values within the range previously recorded in nondeletion α thalassemia traits. Therefore, the observation of mild α globin chain synthesis and/or α globin mRNA deficiency in two mothers is unexpected and suggests that they too may have an atypical type of α thalassemia mutation.

DNA analysis has not so far revealed any abnormality in *cis* to the α globin complexes in any of these patients, and the demonstration that all four α globin genes from patient SW can synthesize mRNA in mouse/human hybrids argues against the presence of conventional α thalassemia mutations. In this regard it is significant that extensive (>1 megabase) deletion of DNA in chro-

mosome band 16p13.3 results in much milder handicap and phenotypic abnormalities (Wilkie et al. 1990): this observation is difficult to reconcile with there being a critical role for a *cis*-acting abnormality in the nondeletion cases. Instead, the alternative *trans*-inactivation hypothesis appears more consistent with the clinical, hematologic, and molecular findings, although direct molecular corroboration of this remains lacking.

There are some interesting parallels between this disorder and the syndrome of acquired Hb H disease. In this rare condition, which usually affects elderly and previously healthy subjects with no prior evidence of α thalassemia, a marked reduction in the synthesis of α relative to β globin results in the appearance of Hb H. Other features of myeloproliferative disease are usually present in this clonal disorder, and overt leukemia sometimes develops, but the Hb H disease itself often fluctuates in severity, and may even remit. The cause of acquired Hb H disease is unknown: there is no evidence of any consistent cytogenetic abnormality (although few cases have been studied carefully), and no molecular derangement of the α globin genes in either the germ line or the bone marrow is detectable, suggesting that abnormality of a *trans*-acting factor could be responsible (Higgs et al. 1983*b*). These observations are reminiscent of the findings in the nondeletion mental retardation cases, and raise the possibility that the mutations causing the two diseases could be equivalent, occurring in the germ line in the mentally retarded patients and somatically in a hematopoietic clone in acquired Hb H disease. An intriguing and currently unexplained feature of acquired Hb H disease is a marked male preponderance—currently 85% of the 47 documented cases (Higgs et al. 1983*b;* and D.R.H., unpublished data).

How could mutation of such a *trans*-acting factor generate the phenotype of Hb H disease, mental retardation and dysmorphic features? Presumably, the mutation would involve a regulatory factor for α globin gene expression, malfunction of which resulted in a decline, but not a complete shutoff, in α globin transcription. There are at least three ways that the other developmental abnormalities could arise in parallel. First, the hypothetical factor might regulate the expression of other important developmental genes unlinked to the α globin locus: promiscuity of action in some of those transcription factors characterized to date is well recognized (Abel and Maniatis 1989). Second, the factor might have an extended local effect on both chromosomes 16 at band 16p13.3 by altering the availability for transcription of the entire domain containing α globin (Zerial et al. 1986). In particular, if it is required for the normal expression of ζ globin in early embryonic life, then a substantial reduction in such expression might cause developmental abnormalities due to tissue hypoxia. Third, the other abnormalities could be due to deletion of a gene(s) contiguous with that encoding the *trans*-acting factor (Schmickel 1986). Of these hypotheses, only the second is currently testable. Study of such features as DNA methylation, DNase I hypersensitivity, and mRNA expression surrounding the α globin complex might reveal evidence of alteration in the nuclear structure and/or expression of adjacent genes. Further characterization of the factor(s) involved in the normal developmental and tissue-specific expression of the α globin complex will be required before these alternative possibilities can be rigorously compared.

Alternatively, a reverse genetic approach might be attempted to prove the existence of such a factor. Are there any clues to the possible chromosomal location of the defect? A variety of circumstantial evidence suggests the X chromosome as the most likely site. Assuming that the boy PE has normal sex chromosomes, all five probands have an XY karyotype, consistent with this hypothesis. Although we cannot identify any definite examples of X-linked inheritance, the two mothers with mild α thalassemia defects could be manifesting carriers of an X-linked recessive trait. Failure to observe any recurrences in siblings may relate to the observation of six healthy sisters, but only one brother, within the five sibships. The unique observation of Hb H cells in the sister of patient SW, in the absence of any other evidence of α thalassemia, could represent a lyonization phenomenon. The striking male bias in acquired Hb H disease is unusual in hematologic malignancy, and is consistent with involvement of a sex-linked factor in this condition.

Does the clinical phenotype suggest similarity with any previously characterized genetic disease? Interpretation is hampered by the rather nonspecific nature of the dysmorphic features. The existence of genital abnormalities in all the nondeletion patients is therefore noteworthy, because the mechanism of this is potentially accessible to study. Several syndromes have been described in which various degrees of genital ambiguity, including male pseudohermaphroditism, and mental retardation may both be present—for instance, the Smith-Lemli-Opitz syndrome (Bialer et al. 1987), Wilms tumor/aniridia/genitourinary anomalies/mental retardation (WAGR complex; Turleau et al. 1984), and testicular regression syndrome (de Grouchy et al. 1985);

however, the clinical features of these syndromes are otherwise quite distinct from the present cases.

In summary, we have defined a new syndrome of Hb H disease, severe mental retardation, genital abnormalities, and dysmorphic features, the cause of which is unknown but which is probably due to a mutation unlinked to the α globin locus. This represents a completely separate phenomenon from the deletion type of α thalassemia/mental retardation syndrome described in the accompanying article (Wilkie et al. 1990), being different at the clinical, hematologic, and molecular levels. Although all cases to date have presented with Hb H disease, we cannot exclude the possibility that a milder phenotype exists. We therefore propose the general term "α thalassemia/mental retardation syndrome (nondeletion type)" to describe this condition. Very little is known at present about the molecular pathology and clinical manifestation of defects in *trans*-acting factors (Reith et al. 1988). If it can be shown that such a defect is indeed responsible for this syndrome, it will serve as an instructive model for the further study of the actions of such factors, as well as the mechanisms of unexplained mental handicap.

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