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Identification of Microdeletions Spanning the Diamond-Blackfan Anemia Locus on 19q13 and Evidence for Genetic Heterogeneity

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Summary

Diamond-Blackfan anemia (DBA) is a rare pure red-cell hypoplasia of unknown etiology and pathogenesis. A major DBA locus has previously been localized to chromosome 19q13.2. Samples from additional families have been collected to identify key recombinations, microdeletions, and the possibility of heterogeneity for the disorder. In total, 29 multiplex DBA families and 50 families that comprise sporadic DBA cases have been analyzed with polymorphic 19q13 markers, including a newly identified short-tandem repeat in the critical gene region. The results from DNA analysis of 29 multiplex families revealed that 26 of these were consistent with a DBA gene on 19q localized to within a 4.1-cM interval restricted by loci D19S200 and D19S178; however, in three multiplex families, the DBA candidate region on 19q13 was excluded from the segregation of marker alleles. Our results suggest genetic heterogeneity for DBA, and we show that a gene region on chromosome 19q segregates with the disease in the majority of familial cases. Among the 50 families comprising sporadic DBA cases, we identified two novel and overlapping microdeletions on chromosome 19q13. In combination, the three known microdeletions associated with DBA restrict the critical gene region to ~1 Mb. The results indicate that a proportion of sporadic DBA cases are caused by deletions in the 19q13 region.

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Introduction

Diamond-Blackfan anemia (DBA; MIM 205900) is characterized by chronic constitutional aregenerative anemia with absent or decreased red cell precursors in the bone marrow but with otherwise normal cellularity (Diamond and Blackfan 1938; Alter 1980; Halperin and Freedman 1980; Young and Alter 1994). Most patients present with anemia in the neonatal period or in infancy. Additional findings frequently include macrocytosis, elevated fetal hemoglobin, and increased red-cell adenosine deaminase (ADA; Glader and Backer 1988). Associated physical anomalies are present in at least 30% of children with DBA, with a wide range of severity. Craniofacial abnormalities are common, with a cleft or high-arched palate, hypertelorism, and a flat nasal bridge (Cathie 1950). Thumb abnormalities are present in 10%–20% of affected children, range in severity from flat thenar eminence to absent radii, and include the classic triphalangeal thumb (Aase and Smith 1969). Other defects frequently observed include atrial or ventricular septal defects, prenatal or postnatal growth retardation, urogenital anomalies, learning difficulties, strabismus, and cataracts (Diamond et al. 1976; Halperin and Freedman 1989; Young and Alter 1994; Ball et al. 1996; Janov et al. 1996). Most cases of DBA are sporadic, with an equal sex ratio, but at least 10% of patients have a positive family history for the disorder with either an autosomal dominant inheritance (Viskochil et al. 1990; Gojic et al. 1990) or an apparently autosomal recessive inheritance (Madanat et al. 1994). There are also several reports of families that show dominant inheritance with incomplete penetrance (Mott et al. 1969; Altman and Gross 1983).

We recently identified a girl with a balanced translocation t(X;19)(p21;q13) associated with the disease

(Gustavsson et al. 1997*a*). On the basis of this observation, we performed a linkage analysis on multiplex DBA families with chromosome 19q markers. The results showed linkage of the DBA phenotype to the chromosomal region with a two-point maximum LOD score (Z_{max}) of 7.08 for D19S197 and no indications of heterogeneity in familial cases (Gustavsson et al. 1997*b*). Moreover, DNA analysis of one patient with sporadic DBA revealed a de novo microdeletion that overlaps with the translocation break point (Gustavsson et al. 1997*b*).

After the identification of the 19q13 region as a carrier for a gene for DBA, additional DBA families were identified. DNA analysis of sporadic DBA cases revealed hemizygosity for a critical gene region on 19q13 in two additional patients. The complex phenotypes associated with the overlapping microdeletions suggest the involvement of genes for mental retardation and skeletal malformations. The DNA-marker analysis of samples from 29 multiplex DBA families showed that in three families linkage was excluded from the entire 19q13 region, which indicates genetic heterogeneity.

Table 1

Two-point LOD Scores in Each Family between DBA and Chromosome 19 Marker Locus D19S197 in Multiplex Families

Family/LOD Score	Family/LOD Score	Family/LOD Score
1/.30	11/.78	21/.26
2/.25	12/.30	22/−.74
3/.26	13/.00	23/.30
4/.83	14/−.74	24/n.i.
5/.26	15/n.i.	25/−.74
6/.82	16/−.74	26/(−3.84)
7/n.i.	17/.26	27/(−5.25)
8/.30	18/(−4.94)	28/.26
9/.56	19/−.18	29/.30
10/.90	20/.30	

NOTE.—Recombination fraction (θ) = .00; Z_{max} = 4.10(−9.93). Table results from linkage analysis by use of a dominant model in all families (penetrance .9). Families 1–13 were reported elsewhere (Gustavsson et al. 1997*b*). LOD scores in parentheses (families 18, 26, and 27) correspond to families I–III (fig. 1) not linked to chromosome 19q13. Families 14, 16, 19, 22, and 25 correspond to families IV–VIII (fig. 2) with possible incomplete penetrance. Families not informative for D19S197 are indicated by “n.i.” In a different linkage analysis, we used a dominant model for families that follow a dominant mode of inheritance and a recessive model for families that follow a recessive inheritance (families 11, 12, 13, and 29). With this combination of either a dominant or a recessive model, Z_{max} = 5.84 was obtained.

Subjects and Methods

Subjects

Blood samples were obtained from unselected DBA patients, their (healthy) siblings, and their parents. Of the 79 families included in this study, 29 families included >1 affected individual, whereas in 50 families cases of DBA were sporadic. Thirteen families (families 1–13, table 1) of the 29 families with familial DBA were included in a previous linkage study (Gustavsson et al. 1997*b*). Four families were consistent with an autosomal recessive inheritance, and 25 families showed an autosomal dominant inheritance. All patients had been diagnosed in pediatric hematology/oncology departments in their country of origin. The minimal diagnostic criteria included normochromic anemia in infancy (<2 years), low reticulocyte counts, absent or decreased bone marrow red-cell precursors (<5% of nucleated cells), and a normal chromosome fragility test (mitomycin C or diepoxybutane). Additional features included the presence of malformations, macrocytosis, elevated fetal hemoglobin, and elevated erythrocyte ADA levels (Glader and Backer 1988). Fetal hemoglobin and ADA were not consistently determined in all family members, because the tests were not available at some hospitals. The patients, with the exception of a few mildly affected family members, were diagnosed before the age of 2 years. Healthy family members aged <2 years were not included in the linkage analysis. A variety of associated malformations were identified among the affected patients, including skeletal anomalies of the hand and forearm, heart defects, cataracts, and short stature. Mental retardation associated with DBA was found in seven sporadic cases. Family members were considered nonaffected if they had normal hemoglobin levels for ⩾2 years, had no history of transient anemia or of transfusion requirements, and had none of the associated physical anomalies, including those revealed by clinical examination of the hands. Coexistence of mild and severe anemia was observed in several multiplex families. One family has previously been described from a clinical point of view (family VII, fig. 2; Mott et al. 1969).

Fanconi anemia was excluded by chromosome fragility test (mitomycin C or diepoxybutane) in at least one affected member per family (frequency of breakage <10%). The same individuals were also karyotyped by conventional cytogenetic studies. One sporadic DBA patient had an apparently balanced de novo translocation, t(8;19)(q35;q13). Lymphoblastoid cell lines were established for further genetic analysis from the peripheral blood of a few patients by transformation with the Epstein-Barr virus.

Identification of a Novel Microsatellite Marker

Sequences of genes assigned to the D19S197–D19S408 interval (Ashworth et al. 1995) were analyzed for the presence of di- and trinucleotide repeats through GenBank. The DNA sequences were analyzed for short tandem repeat units. Flanking primers were designed in order to generate specific amplicons. PCR products and the presence of polymorphisms were detected as de-

scribed below (DNA analysis). The distribution of alleles was determined from the analysis of DNA from 20 unrelated healthy individuals.

DNA Analysis

Genomic DNA was extracted from peripheral blood in accordance with standard procedures. The polymorphic dinucleotide repeats D19S200, D19S197, LIPE, D19S408, and D19S178, assigned to the 19q13 region (Dib et al. 1996), were amplified from the genomic DNA of all families, as described elsewhere (Gustavsson et al. 1997*b*). A newly identified polymorphic repeat, PG1 (GenBank accession number L32754), is located within the critical DBA gene region between markers D19S197 and LIPE. The PG1 repeat was amplified from the genomic DNA of the families with the specific primers: 5′-TGATGTTGCCACAGCACTTC, forward, and 5′-CTCTCTGAGTCTACAACCAG, reverse. For the analysis of all microsatellites, PCR was performed at optimized conditions for genomic DNA by the use of primers end-labeled with $\gamma[^{32}P]$-ATP. The PCR reactions were performed in 96 well microtitre plates, in a reaction volume of 10 μl, with 20 ng of genomic DNA. The PCR mixture contained 2 pmol of each primer, 0.1 mM of each of the four deoxytrinucleotide triphosphates, and 0.5 U of *Taq* DNA polymerase. The PCR conditions were 4 min at 94°C for denaturing, followed by 28 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C. PCR products were separated on polyacrylamide gels by electrophoresis and were visualized by autoradiography. The genotypes and the haplotypes were assigned manually.

Linkage Analysis

Linkage analysis was performed in 29 multiplex families, 13 of which were included in a previous study (Gustavsson et al. 1997*b*). LOD scores were calculated in two separate ways. In the first analysis, all families were considered dominant. In a second analysis, the families who followed a recessive mode of inheritance ($n = 4$) were analyzed separately under a recessive model. Different penetrant models (.7–.9) were used and the disease-gene frequency was set to 10^{-6} with no sex difference. Allele frequencies were taken from the Genome Database (GDB) when possible. The order and distances between the chromosome 19 markers were determined from maps from Généthon (Dib et al. 1996), the chromosome 19 workshop (Mohrenweiser et al. 1996), Lawrence Livermore National Laboratory (Ashworth et al. 1995), and GDB. The order of marker loci is centromere-D19S200-D19S197-PG1-LIPE-D19S408-D19S178-telomere. Two-point LOD scores were performed by use of FASTLINK version 3.0P (Lathrop et al. 1984) from the original LINKAGE analysis computer package (Ott 1991).

FISH Analyses of Microdeletions

Chromosome preparations from three DBA patients that showed constitutional microdeletions for the 19q13 region were analyzed by FISH. The deletion found in patient RG was described elsewhere (Gustavsson et al. 1997*b*). FISH to metaphase chromosomes was performed essentially as described elsewhere (Lichter et al. 1988; Gustavsson et al. 1997*a*). Cosmid DNA was labeled either with biotin or digoxigenin using nick translation. Hybridization of probes was detected by the application of a single layer of FITC-avidin (Vector labs) or rhodamine-labeled antidigoxigenin (Boehringer Mannheim). Double hybridization was detected by means of a mixture of FITC-avidin and rhodamine-labeled antidigoxigenin. Chromosomes were counterstained with DAPI (Serva) and mounted in antifade solution (Vector labs). The slides were analyzed with a Zeiss Axioskop epifluorescence microscope, and the images were merged by use of a CCD camera (Photometrics) and the Quips SmartCapture FISH software (Vysis).

Cosmids

A physical map has been constructed over the 19q13 region (Ashworth et al. 1995; Mohrenweiser et al. 1996). The chromosome 19 cosmids used were obtained exclusively from Lawrence Livermore National Laboratory with their corresponding numbers (Ashworth et al. 1995). In total, seven cosmids were used for FISH analyses, and their relative order from the centromere to the telomere is as follows: 9476 (RYR1), 16767 (CYP2A12), 14353 (CGM1), 24450 (ATP1A3), 19343 (D19S336), 9933 (CGM9), and 8764 (XRCC1).

Results

Identification of Polymorphic Short Tandem Repeats

One short tandem repeat was identified by computer search of the genomic sequences assigned between markers D19S200 and D19S408. The repeat is localized in intron 4 of the gene-encoding IGA (CD79a), which maps between D19S197 and LIPE. The repeat is complex and consists of a combined dinucleotide and a trinucleotide repeat (GenBank accession number L32754). The repeat, designated PG1, was analyzed for polymorphisms on the DNA from 20 independent individuals. Separation of PCR products revealed alleles with a range of 186–218 bp. The degree of heterozygosity was .85 from 20 chromosomes.

Haplotype Analysis of Familial DBA Cases

The most likely haplotypes from the six chromosome 19 markers were constructed when possible. The results

from 26 of 29 multiplex families were consistent with a DBA gene localization on chromosome 19q between loci D19S200 and D19S178. In three multiplex families, none of the inherited marker alleles were shared by the affected family members, respectively (fig. 1). The chance for a double recombination between two adjacent markers in the critical region is <.1%. Thus, the most likely explanation for the results is a DBA locus not localized to 19q13.2 in these families. Affected individuals of the three nonlinked families presented elevated levels of ADA. The clinical picture was typical for DBA (diagnostic criteria included age of onset, bone marrow biopsies, reticulocyte counts, and chromosome fragility tests) and was similar to patients from families in which the 19q markers cosegregate with DBA. Fetal hemoglobin measurements were available only from affected individuals of family I (fig. 1), and the levels were found to be elevated. With the exception of families I–III, no evidence for genetic heterogeneity was found; however, in five families with a dominant mode of inheritance for DBA, the disease-associated chromosome 19 haplotype was also transmitted to a healthy family member (>2 years and normal hemoglobin levels; fig. 2). The levels of erythrocyte-ADA activity were measured in three of these healthy siblings, and the activity was found to be elevated in one of them (family V, ind. II:2; fig. 2). The results from ADA measurements suggest an incomplete clinical penetrance in this apparently healthy sibling. The results obtained from the analysis of families IV and VI–VIII could be explained either by an incomplete penetrance for the disorder or by genetic heterogeneity. In one family (family VIII; fig. 2), germinal mosaicism for a mutant 19q allele is a third possible explanation for the transmission of identical haplotypes from the healthy woman in the first generation to her affected and healthy children, respectively.

Linkage Analysis

The six chromosome 19q markers D19S200, D19S197, PG1, LIPE, D19S408, and D19S178 were analyzed. LOD scores were first calculated by use of an autosomal dominant model for all familial cases (table 1). A two-point linkage analysis revealed Z_{max} = −9.93 at D19S197, with an assumed penetrance of .9. By the exclusion of the three nonlinked families (family I, family II, and family III; fig. 1) from the analysis, a pairwise Z_{max} at D19S197 was obtained. Z_{max} was further increased to 5.16 by reduction of the penetrance from .9 to .7.

A second calculation used a dominant model for families that showed a dominant inheritance (n = 25) and a recessive model for families with an apparently recessive inheritance (n = 4). With the assumption of this combination of either dominant or recessive inheritance and a penetrance of .9, a two-point Z_{max} = 5.84 was obtained (dominant families, 2.70; recessive families, 3.14). No evidence for heterogeneity was found with the HOMOG option from the original LINKAGE analysis computer package (Lathrop et al. 1984).

Haplotype Analysis of Sporadic DBA Cases

In 50 families that included a sporadic case of DBA, parents and at least one healthy sibling were analyzed for the segregation of 19q marker alleles at loci D19S200, D19S197, PG1, LIPE, D19S408, and D19S178. Apparent loss of parental alleles was observed in three affected individuals. The three patients presented with common clinical features in addition to DBA, including mental retardation and skeletal malformations of the long bones and the spine. Patient MH, who carries an apparent balanced translocation t(8;19)(q35;q13), was hemizygous for the microsatellite loci D19S197 and PG1 with loss of the paternal allele. In patient HC, the

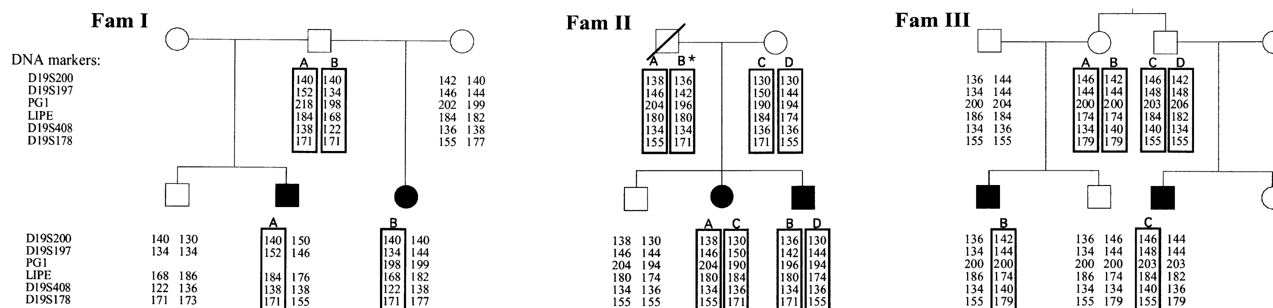

Figure 1 Three multiplex DBA families with haplotypes spanning the candidate gene locus on chromosome 19q13.2. DNA marker loci are shown, *left*, with relative marker order from centromere to telomere. Corresponding marker alleles and haplotypes are boxed and indicated below each symbol. In each kindred, no common haplotype was inherited by affected relatives, thereby indicating genetic heterogeneity. Asterisks (*) indicate deduced haplotypes.

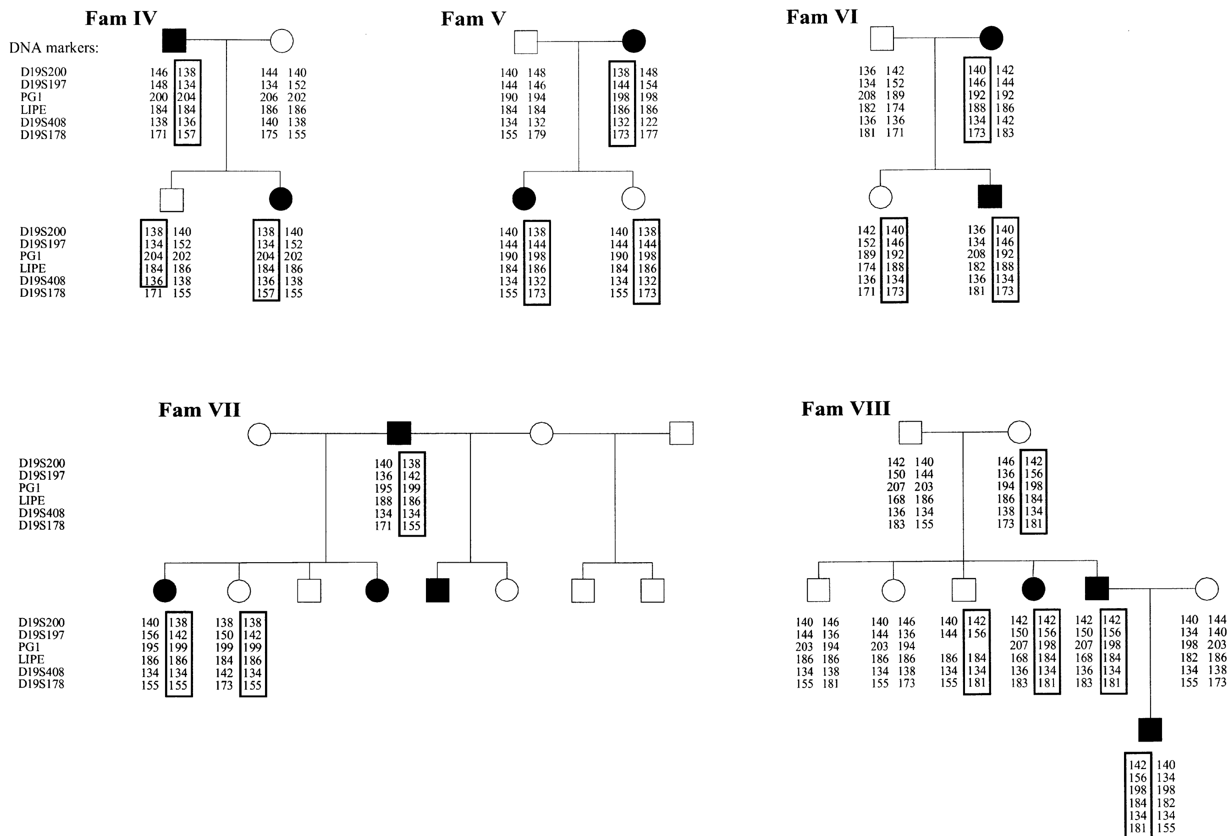

Figure 2 Segregation of 19q marker alleles in families with dominantly inherited DBA. In each of the five different families, apparently healthy family members have inherited the disease-associated chromosome 19q haplotype, which suggests incomplete penetrance or locus heterogeneity. The activity of erythrocyte ADA was measured in families V, VI, and VII; increased ADA level was found in one healthy sibling (family V, ind. II:2). Normal ADA levels were found in healthy siblings of family VI (individual II:1) and family VIII (individual II:3).

markers D19S197, PG1, and LIPE revealed loss of the maternal alleles. In patient RG, markers PG1 and LIPE revealed loss of the paternal alleles (fig. 3). The segregation of marker alleles was analyzed in 93 healthy siblings of patients with sporadic DBA. Forty-eight healthy siblings shared one inherited 19q13 haplotype with the proband, 23 shared both 19q13 haplotypes, and 22 shared none. The results indicate an almost random segregation of marker alleles on chromosome 19q to healthy siblings of sporadic DBA cases.

Mapping the Microdeletions by FISH

FISH analyses of metaphase chromosomes of the three patients with loss of parental marker alleles confirmed hemizygosity for part of the 19q13.2 region (fig. 4). The extremities of the deletions were approximately localized with cosmids derived from a chromosome 19 map (Ashworth et al. 1995; Mohrenweiser et al. 1996). The deletion associated with t(8;19)(q35;q13) in patient MH was mapped between cosmid 16767 on the centromeric side and 24450 on the telomeric side. Likewise, the deletion identified in patient HC was found to extend between cosmids 16767 and 9933. The microdeletion in patient RG was previously shown to extend between cosmids 16923 (TGFB1) and 8764 (XRCC1) (Gustavsson et al. 1997*b*). The proximal breakpoint of the deletion was further restricted by cosmid 16767. In summary, the three deletions define a common overlap restricted by cosmid 16767 (CYP2A12) on the centromeric side and cosmid 19343 (D19S336) on the telomeric side (fig. 4). The two cosmids are separated by ~1 Mb of DNA (Ashworth et al. 1995), which narrows the DBA candidate region from 1.8 Mb (Gustavsson et al. 1997*b*).

Discussion

We previously localized a gene for DBA to a 1.8-Mb region, with indications for homogeneity (Gustavsson et al. 1997*a*, 1997*b*). Although a majority of familial cases in this study are consistent with a locus on chromosome 19q13.2, the results from segregation of 19q13 marker alleles in three families cannot be explained by a primary

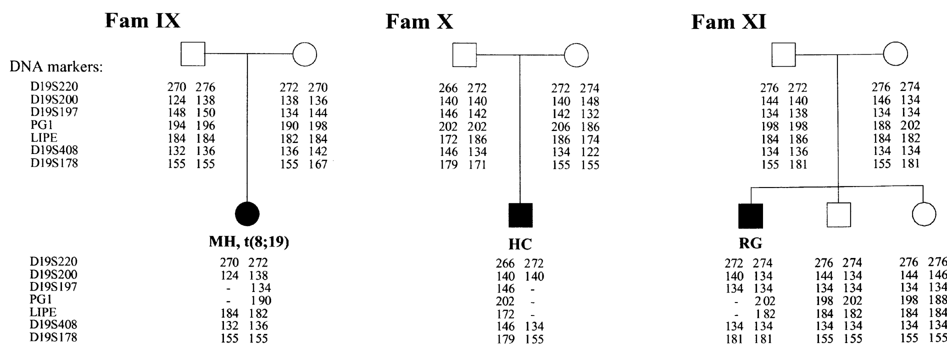

Figure 3 Identification of microdeletions in the 19q13.2 region in three sporadic cases of DBA. Haplotypes reveal hemizygosity on the paternally derived chromosome of patient MH and patient RG. Patient MH shows loss of markers D19S197 and PG1; patient RG shows loss of markers PG1 and LIPE. Patient HC is hemizygous for markers D19S197, PG1, and LIPE, with loss of the maternally derived alleles. Patient RG was presented elsewhere (Gustavsson et al. 1997*b*).

gene defect in this region. Our results indicate the existence of at least two genes for DBA. The variable inheritance in familial cases with either a dominant or a recessive pattern, in combination with a variety of clinical abnormalities, has previously led to the suggestion of genetic heterogeneity for the disorder. This suggestion has also been supported by in vitro experiments (Tsai et al. 1989).

When the chromosome 19q13.2 haplotype in each sporadic patient with DBA was compared with the haplotype inherited by 93 healthy siblings, the results showed an almost random distribution of inherited alleles. This contradicts a recessive inheritance for a gene on 19q13 and could be explained by new mutations of a dominant gene on 19q, incomplete penetrance for a dominant gene, or genetic heterogeneity. The three overlapping microdeletions on 19q13 found among the 50 sporadic cases analyzed indicate that de novo rearrangements are one cause of the disease in sporadic cases.

The size of the common overlap of the microdeletions is ~1 Mb, and the region spans >10 known genes (Ashworth et al. 1995; Mohrenweiser et al. 1996). The cause of DBA is not known, and the large number of genes mapped to the deleted interval does not allow us yet to specify what gene is responsible for the disease. In addition, there are several expressed sequence tags mapped to 19q13.2. It is interesting to note that several gene families are clustered in the region that corresponds to the microdeletions. At least six copies of the cytochrome P-450 gene family, which are hemoproteins involved in the metabolism of steroids and the detoxification of drugs (Nebert 1991), are clustered in the vicinity of the proximal breakpoints. In a more telomeric direction, the carcinoembryonic antigen (CEA) gene family, which includes the CEA gene-family members and the pregnancy-specific glycoprotein gene families, are represented by several copies (Thompson et al. 1992; Teglund et al. 1994). The CEA gene family encodes a large family of glycoproteins with an unknown function. Tandemly repeated and homologous sequences may be predisposed to homologous unequal recombination. Such events have been suggested to be the mechanisms for microdeletions in chromosome 17p11.2 associated with Smith-Magenis syndrome (Chen et al. 1997); however, a similar mutation mechanism needs to be confirmed by DNA sequencing of the deletion breakpoints in our patients.

A substantial proportion of patients with DBA have associated features, in addition to anemia. Common associated malformations affect the hand and the thumb, but skeletal abnormalities of the head have also been observed. These phenotypes may result from contiguous deletions that involve several genes or from mutations in a single gene important both for hematopoiesis and skeletal development. We identified three patients with overlapping deletions associated with skeletal abnormalities that affect the extremities, the skull, and the spine, as well as mental retardation, which has been reported previously in some patients with DBA (Janov et al. 1996). These overlapping deletions suggest the involvement of several genes as the cause of the phenotype of patients MH, HC, and RG. Further molecular studies of patients with complex features and DBA may clarify whether their phenotypes are caused by microdeletions.

Our combined results suggest genetic heterogeneity for DBA, of which the majority of multiplex families appear linked to a gene on chromosome 19q. In addition, a proportion of sporadic cases result from new mutations of a gene on 19q13.2, of which three de novo microdeletions indicate a critical gene region for DBA. The detailed mapping of these deletions may indicate candidate genes for overlapping phenotypes that include mental retardation and skeletal malformations. Further linkage studies in families not linked to chromosome

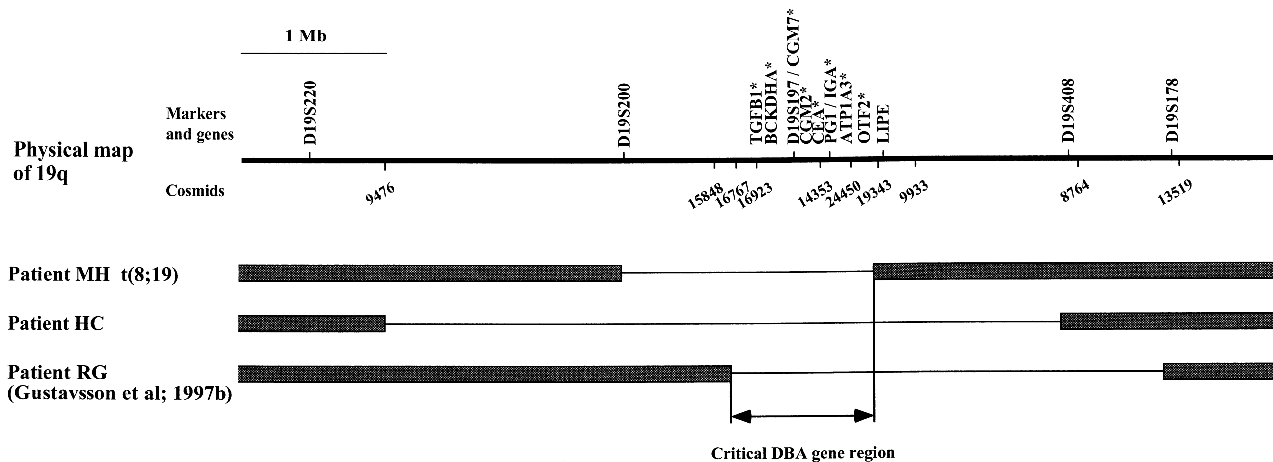

Figure 4 Continuous physical map of part of the 19q13.2 region (*top*); includes known markers and genes found on a previously presented physical map (Ashworth et al. 1995). Asterisks (*) indicate transcribed genes in the critical interval. The extent of the three microdeletions are shown (*below*). Horizontal bars represent chromosome regions present in the three patients. The deleted regions in each patient are indicated by an intervening line, respectively. The critical DBA gene region, indicated by a two-headed arrow (⟷), is restricted by cosmids 16767 and 19343.

19q13 are required in order to identify a second DBA locus. We suggest that in certain multiplex families, and in sporadic families in general, great care should be taken when using polymorphic 19q markers for diagnosis and genetic counseling.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Web/Search (accession number L32754, CD79a gene)

Généthon, http://www.genethon.fr (for D19S408 and D19S178)

Genome Database, http://gdbwww.gdb.org (for D19S200, D19S197, LIPE, D19S408, and D19S178)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for DBA [MIM 205900])

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