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Genetic characterization of DiGeorge and related syndromes associated with 22q11.2 deletions

By

Suzanne Demczuk

Department of Biology

McGill University

Montréal, Québec, Canada

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Genetic characterization of DiGeorge syndrome.

Abstract

DiGeorge syndrome (DGS) is a developmental defect associated with deletions in chromosomal region 22q11.2. Recently, other syndromes (Velo-Cardio-Facial syndrome, Conotruncal Anomaly Face syndrome, isolated conotruncal cardiopathy) with overlapping phenotypes have been found to be associated with deletions of a similar extent in this chromosomal region. All these syndromes have been grouped under the acronym CATCH 22 (Cardiac defect, Abnormal facies, Thymic hypoplasia, Cleft palate, Hypocalcemia, chromosome 22q11.2 deletions). In order to characterize genetically this group of syndromes, we have searched for deletions in the 22q11.2 chromosomal region by fluorescence *in situ* hybridization (FISH). A set of 6 cosmid probes dispersed within the whole length of the DGS deleted region was used to screen 23 patients. A 22q11.2 deletion was observed in 96% of the patients studied. Furthermore, there does not seem to exist any correlation between the size of the deletion and the phenotype observed, since the majority of patients studied, although widely divergent in their clinical manifestation of DGS, appeared to present the same extent of deletion in this genomic region.

There appears to be a predominance of deletion-bearing mothers in familial CATCH 22 when published pedigrees are examined. Furthermore, our own familial cases and the sporadic cases where the parental origin of the deletion could be deduced using a chromosome 22 short arm heteromorphisms seem to confirm this tendency. Because we had isolated a CA-repeat locus mapping within the DGS deleted region, the parental origin of the deletion in sporadic DGS/VCFS cases was studied by assessing the inheritance pattern of this microsatellite marker. The deleted portion of chromosome 22 was of maternal origin in 16 out of 22 cases (72%). When cases of sporadic, familial and unbalanced translocation inheritance reported in the literature were pooled with these results, there appears to be a net tendency for the deletions to be of maternal origin in CATCH 22 (70 deletions of maternal origin, 21 of paternal origin, $X^2=26.4$, $p<0.0001$).

In order to identify the molecular defect underlying DGS, we embarked on a positional cloning approach. A detailed physical map of the 22q11.2 region was made using one- and two-color FISH on metaphases and G_0 interphase nuclei, and by hybridization to a chromosome 22 hybrid panel. This permitted delineation of a critical region, within which the breakpoint of a balanced translocation carrier affected with DGS was mapping. This breakpoint was cloned by the construction of cosmid contigs, and a novel gene mapped to this region was isolated. The gene potentially encodes an adhesion receptor, and is not interrupted by the balanced translocation breakpoint. Possible mechanisms through which this gene can be involved in the pathogenesis of DGS are presented.

This research project has contributed toward the understanding of the genetics of DGS and related syndromes. Furthermore, a candidate gene for the CATCH 22 syndromes has been isolated and further work will confirm whether it plays a major role in the pathogenesis of these syndromes.

Résumé

Le syndrome de DiGeorge (DGS) est une pathologie du développement associée à des microdélétions de la région chromosomique 22q11.2. Des microdélétions impliquant la même région chromosomique ont été retrouvées chez des patients atteints de syndromes phénotypiquement apparentés, ce qui a permis le regroupement de ces syndromes sous l'acronyme CATCH 22 (Cardiac defect, Abnormal facies, Thymic hypoplasia, Cleft palate, Hypocalcemia, chromosome 22q11.2 deletions). Une étude par hybridation *in situ* fluorescente (FISH) avec 6 sondes cosmidiques localisées dans la région DGS communément délétée a permis de démontrer que 23 sur 24 (96%) patients étudiés présentaient une délétion dans cette région chromosomique. L'étude de la taille des délétions n'a pu mettre en évidence de corrélation entre l'étendue de la perte chromosomique et le phénotype observé, puisque presque tous les patients présentaient une délétion de même taille, mais un phénotype très variable.

Il semble y avoir une prédominance de mères porteuses de délétion dans les cas familiaux de CATCH 22 publiés. De plus, les familles CATCH 22 recrutées au laboratoire et les cas sporadiques où l'origine parentale de la délétion a pu être déduite à l'aide d'un hétéromorphisme du bras court du chromosome 22 semblent confirmer cette tendance. Puisque nous avions isolé un locus microsatellite localisé dans la région DGS communément délétée, l'origine parentale de la délétion a été déterminée chez des cas sporadiques de DGS/VCFS. Cette étude nous a permis de constater que la délétion était d'origine maternelle dans 16 cas sur 22 (72%). En ajoutant à ces données les cas publiés de CATCH 22 sporadiques, familiaux ou survenant à la suite d'une translocation déséquilibrée héritée, il semble y avoir une origine préférentiellement maternelle de la délétion. (70 délétions d'origine maternelle, 21 d'origine paternelle, $X^2=26.4$, $p<0.0001$).

Dans le but d'identifier le ou les gènes impliqué(s) dans la pathogénèse du DGS, une approche de clonage positionnel a été adoptée. Une carte physique détaillée de la région DGS a été établie

en utilisant les techniques de FISH mono- et bicolore sur métaphases et sur noyaux G_0 interphasiques et par hybridation sur un panel d'hybrides somatiques du chromosome 22. Ce travail a permis de délimiter une région critique dans laquelle, justement, est localisé le point de cassure d'une translocation équilibrée retrouvée chez une patiente atteinte de DGS. Le point de cassure de cette translocation a été franchi par l'établissement de contig de cosmides et un nouveau gène a été isolé dans cette région génomique. Ce gène pourrait potentiellement coder pour un récepteur d'adhésion cellulaire, et n'est pas interrompu par le point de cassure. Des mécanismes possibles par lesquels ce gène pourrait être impliqué dans la pathogénèse du CATCH 22 sont présentés.

Ce projet de recherche a contribué à une meilleure compréhension de la génétique du DGS et des syndromes apparentés. De plus, un gène candidat pour le CATCH 22 a été isolé et un travail ultérieur permettra de démontrer si ce gène joue un rôle majeur dans la pathogénèse de ces syndromes.

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List of Abbreviations

BSS: Bernard-Soulier syndrome
CATCH 22: Cardiac defect, Abnormal facies, Thymic hypoplasia, Cleft palate, Hypocalcemia, chromosome 22q11.2 deletions
CTAF: Conotruncal Anomaly Face syndrome
cDNA: Complementary DNA
CHARGE association: Coloboma of the eye, Heart defect, choanal Atresia, Retarded growth/development, Genital hypoplasia, Ear anomalies
CMT1: Charcot-Marie-Tooth disease type 1
COMT: Catechol-O-Methyl transferase
DGA: DiGeorge anomaly
DGCR 2: DiGeorge syndrome critical region gene 2
DGCR: DiGeorge syndrome critical region
DGS: DiGeorge syndrome
DIRVISH: Direct visualisation hybridization
FISH: Fluorescence *in situ* hybridization
FSHD: Facioscapulohumeral muscular dystrophy
GpIb: Glycoprotein Ib
IGF2: Insulin-like growth factor 2
IGLC: Immunoglobulin light chain
kb: Kilobase
kD: Kilodalton
LDLR: Low-density lipoprotein receptor
LRP: Low-density lipoprotein receptor related protein
Mb: Megabase
MDS: Miller-Dieker syndrome
PCR: Polymerase chain reaction
PEG: Polyethylene glycol
PFGE: Pused-field gel electrophoresis
RFLP: Restriction fragment length polymorphism
SSC: sodium chloride/sodium citrate
TUPLE 1: Tup-like/Enhancer of split gene 1
VCFS: Velo-Cardio-Facial syndrome
YAC: Yeast artificial chromosome

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Preface

In compliance with the Faculty of Graduate Studies and Research, the following excerpt from the "Guidelines Concerning Thesis Preparation" is cited below:

"The candidate has the option, subject to the approval of their department, of including as part of the thesis the text, or duplicated published text, of an original paper or papers. Manuscript-style theses must still conform to all other requirements explained in the Guidelines Concerning Thesis Preparation. Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail (e.g. in appendices) to allow clear and precise judgement to be made of the importance and originality of the research reported. The thesis should be more than a mere collection of manuscripts published or to be published. It must include a general abstract, a full introduction and literature review and a final overall conclusion. Connecting texts which provide logical bridges between different manuscripts are usually desirable in the interest of cohesion. It is acceptable for theses to include, as chapters, authentic copies of papers already published, provided these are duplicated clearly and bound as an integral part of the thesis. In such instances, connecting texts are mandatory and supplementary explanatory material is always necessary. Photographs or other materials which do not duplicate well must be included in their original form.

While the inclusion of manuscripts co-authored by the candidate and others is acceptable, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent, and supervisors must attest to the accuracy of the claims at the Ph.D. Oral Defense. Since the task of the Examiners is made more difficult in these cases, it is in the candidate's interest to make the responsibilities of authors perfectly clear."

The work described in Chapter 2, 3, 4 and 5 of this thesis has been/will be published in the following journals:

Chapter 2 Demczuk S., Desmaze C., Aikem M., Prieur M., LeDeist F., Sanson M., Rouleau G., Thomas G. and Aurias A. (1994). Molecular cytogenetic analysis of a series of 23 DiGeorge

syndrome patients by fluorescence *in situ* hybridization. Ann. Genet. 37:60-65.

Chapter 3 Demczuk S., Lévy A., Aubry M., Croquette M.F., Philip N., Prieur M., Sauer U., Bouvagnet P., Rouleau G.A., Thomas G. and Aurias A. Excess of deletions of maternal origin in the DiGeorge/Velo-Cardio-Facial syndromes. A study of 22 new patients and review of the literature. Hum. Genet. *In press.*

Chapter 4 Demczuk S., Delattre O., Vignal A., Weissenbach J., Thomas G. and Aurias A. Physical mapping of 30 CA-repeats on human chromosome 22. Short communication in Genomics, *In press.*

Chapter 5 Demczuk S., Aledo R., Zucman J., Delattre O., Desmaze C., Dauphinot L., Jalbert P., Rouleau G.A., Thomas G. and Aurias A. Cloning of a balanced translocation breakpoint in the DiGeorge syndrome critical region and isolation of a novel potential adhesion receptor gene in its vicinity. *In press.*

The work performed for each of these publications is entirely my own with the following exceptions:

Chapter 2: C. Desmaze and M. Aikem did some of the FISH experiments on a few patients. M. Prieur recruited the patients at the Necker Hospital for Sick Children and performed the karyotypic analysis. F. LeDeist did the immunological evaluations for the patients. M. Sanson isolated the 3F4 Not I linking clone in the laboratory.

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INTRODUCTION

DiGeorge syndrome (DGS) is a developmental defect of the third and fourth pharyngeal pouches which associates hypo- or aplasia of the thymus and parathyroids, and conotruncal cardiac malformations. The etiological factor in a great majority of DGS cases is monosomy for the 22q11.2 chromosomal region either through a large interstitial deletion of that region (inherited or de novo) or through an unbalanced translocation involving chromosome 22 (inherited or not).

More recently, deletions involving the same chromosomal region as DGS have been reported for other closely related syndromes such as the velo-cardio-facial syndrome (VCFS), isolated conotruncal cardiac defects (either sporadic or familial) and the conotruncal anomaly face syndrome (CTAF), which prompted the coining of the acronym CATCH 22 (Cardiac defect, Abnormal facies, Thymic hypoplasia, Cleft palate, Hypocalcaemia, chromosome 22q11 deletions) to designate this group of related syndromes. The CATCH 22 group of syndromes appears to be a major cause of birth defects with a minimum prevalence estimated to 1 in 4000. In fact, deletions in 22q11.2 may be etiological in 5% of all newborns with heart defects (Wilson et al., 1994). It thus appears that there is/are very important gene(s) playing a role in embryological development in this chromosomal region.

DGS was originally thought to be a contiguous gene syndrome. However, it has not been possible to correlate the extent of 22q11.2 deletions with the presence or the severity of a phenotypic feature. In contrast, one single observation of a mother and daughter both bearing a balanced t(2;22) (q14.1;q11.1) translocation and displaying DGS/VCFS phenotypes has been reported and suggests that the translocation breakpoint lies within the major DGS locus. In addition, a few patients do not carry any deletions or chromosomal aberrations in 22q11.2, suggesting that these cases could be due either to very small deletions or point mutations within one major gene causing DGS, or to another etiology.

The aim of this study was to characterize genetically the DGS region. Probes mapping to the proximal part of chromosome 22 were used to search for deletions in DGS patients with apparently normal

karyotypes. *Chapter 2* reports the screening of a series of 23 DGS patients by fluorescence *in situ* hybridization (FISH) with a set of 6 cosmid probes dispersed within the whole length of the DGS deleted region. This molecular cytogenetic approach seemed the most appropriate for deletion detection, because absence of a fluorescent signal on one chromosome 22 can be directly observed on metaphase chromosomes. Conversely, confirmation of deletions by Southern hybridization requires DNA from the patients' parents, and probes with a high degree of polymorphism or quantitation of the hybridization signal.

From published data, it does not appear that the variable expressivity of the phenotypic manifestations in cases of CATCH 22 is correlated with the extent of the 22q11.2 deletions. In contrast, the concept that the parental origin of the deletion could modulate the variable expressivity of the phenotype has been proposed before and seems very attractive. Because we possessed the only CA-repeat mapping within the deleted region, we chose to verify this hypothesis. *Chapter 3* reports the determination of the parental origin of the deleted chromosome 22 in sporadic DGS by assessing the inheritance pattern of this microsatellite locus, and by using a cytogenetic heteromorphism on the short arm of chromosome 22.

In order to identify the molecular defect underlying DGS, we embarked on a positional cloning approach. First, the region was saturated with new probes, and the precise mapping of the corresponding loci was done using the various tools available in the laboratory. Probes were first mapped by Southern blot hybridization to a chromosome 22 hybrid panel that divides this chromosome in 28 subregions and the DGS deleted region in 4. Alternatively, physical mapping of CA-repeat loci was performed by PCR amplification of DNA from this hybrid panel with primers flanking these sequences. Thirty microsatellite markers were assigned to subregions of chromosome 22 and these physical mapping data are reported in *Chapter 4.*

Whenever a probe was mapped within the DGS deleted region, the corresponding cosmid(s) were searched by hybridization to a chromosome 22-enriched cosmid library arrayed in microtitration

plates (25 000 clones, 8 genome-equivalents). The technique of FISH was then used to order these probes relative to 9 translocation breakpoints borne by DGS patients and that divide the DGS deleted region in 5 further subgroups. If more than one cosmid mapped between two translocation breakpoints, two-color FISH was used to order these loci relative to each other. A detailed physical map of the proximal part of chromosome 22 was thus obtained, and a minimal candidate interval within which the DGS gene(s) would be contained was defined.

Physical distance between markers flanking the breakpoint that presumably disrupts the DGS gene was estimated by two-color FISH on interphase nuclei, and because this distance appeared relatively small, a chromosome walk was undertaken in order to clone the t(2;22) balanced translocation breakpoint. Two cosmid contigs were constructed (170 kb and 325 kb, respectively), and the balanced translocation breakpoint was crossed. *Chapter 5* describes the cloning of this breakpoint and the isolation of a gene mapping in its vicinity.

CHAPTER 1

LITERATURE REVIEW

1. DiGeorge Syndrome

1.1 Clinical aspect

It was in 1965 that Dr. Angelo DiGeorge presented for the first time 3 infants who at autopsy showed congenital absence of the thymus and parathyroids. These cases illustrated the two component concept of the immune system proposed by Cooper et al. (1965). This work derived from experiments on chicks, revealed that ablation of the bursa caused agammaglobulinemia, with full capacity to retain cellular immunity, while thymectomy lead to a defective cellular immunity with intact ability to produce immunoglobulin. The 3 infants reported by DiGeorge (1965) therefore, represented the human counterpart of thymectomized chicks. Furthermore, DiGeorge presented a 4th, still alive, infant with congenital hypoparathyroidism, on whom immunologic studies could be done. The patient fitted with the hypothesis of Cooper et al. (1965) in that he had defective cellular immunity (e.g. failure to reject a homologous skin graft, delayed hypersensitivity response through a skin test), but normal circulating lymphocytes, plasma cells in lymph nodes and circulating immunoglobulins. The child was correctly predicted to have no thymus (DiGeorge, 1968), and congenital hypoparathyroidism with absence of the thymus became dubbed "DiGeorge syndrome".

The concomitant absence of thymus and parathyroids is logical since they both share a common embryologic origin, namely the 3rd and 4th pharyngeal pouches (Harvey et al., 1970). In fact, this pathology had been observed before DiGeorge's report, under the name "III-IV pharyngeal pouch syndrome" (Lodbell, 1959; Taitz et al., 1966). Following DiGeorge's observations, an additional 20 cases of DiGeorge/3rd-4th pharyngeal pouch syndrome were reported in the literature, and in 1972, Lischner, recognizing the phenotypic variability of this clinical condition, proposed some categorizations: 1) the III-IV pharyngeal pouch syndrome would include hypoplasia or absence of the thymus and/or parathyroid glands accompanied by another congenital malformation, particularly conotruncal heart

defects and facial dysmorphism; 2) complete DiGeorge syndrome would be designated as those cases of III-IV pharyngeal pouch syndrome with no thymic remnants; whereas 3) partial DiGeorge syndrome would be composed of thoses cases in which some thymic tissue is present, but the thymus is small or there is defective cell-mediated immunity.

The association of DGS with congenital cardiac malformations, particularly anomalies of the great vessels of the heart was further documented (Harvey et al., 1970; Freedom et al., 1972; Finley et al., 1977). Conley et al. (1979), in a retrospective study of 25 DGS patients, confirmed this association: all the patients had cardiovascular anomalies on autopsy and congenital heart disease was the presenting complaint in 60% of cases. In addition, 60% of patients had an abnormal facies, 4 had cleft lip or palate or bifid uvula and 3 had an associated congenital defect (e.g. Zellweger syndrome, CHARGE association, holoprosencephaly).

In view of the broad spectrum of severity observed in DGS, Müller et al. (1988; 1989) in a study of 16 prospectively ascertained DGS cases, devised a clinical index based on phenotype and laboratory tests which could permit the distinction between partial and complete forms of the disease. However, the scores obtained using this index were not weighted, and some clinical observations may be more pertinent to DGS than others, or not independent from one another (Greenberg, 1993). Consequently, this index never got into general use.

From these studies, it can be seen that even a few years after the first description of the syndrome, investigators were surprised by the clinical variability of DGS, in terms of the type of and the degree to which structures are affected (Freedom et al., 1972; Conley et al., 1979; Müller et al., 1988; Greenberg, 1989). What are then, the clinical features delimiting DiGeorge syndrome? Table 1 displays the various clinical findings reported in the large series of DGS individuals studied. Conotruncal cardiac defect is a feature found in most studies, especially a strong association is seen between specific types of cardiac defects (e.g. interrupted aortic arch, truncus arteriosus, right sided aortic arch, tetralogy of Fallot) and DGS. It is

Table 1

Frequency of the clinical features observed in the DGS series studied

Author (year)	Cardiac defect No. (%)	HypoCa/ Parathyroids No. (%)	Thymus/ Immunodef[1] No. (%)	Facial Dysmorph.[2] No. (%)	Other Malform.[3] No. (%)
Freedom et al. (1972)	19/23 (83)	22/23 (96)	22/23 (96)	10/23 (43)	8/23 (35)
Conley et al. (1979)	25/25 (100)	17/23 (74)	24/25 (96)	ND	19/25 (76)
Durandy et al. (1986)	15/17 (88)	17/17 (100)	10/17 (59)	14/17 (82)	4/17 (24)
Van Mierop et al. (1986)	50/50 (100) *	20/22 (90)	45/50 (90)	19/50 (38)	18/50 (36)
Müller et al. (1988)	14/16 (88)	11/16 (69)	13/16 (81)	16/16 (100)	7/16 (44)
Greenberg et al. (1988a)	25/28 (89)	26/28 (93)	28/28 (100)	14/28 (50)	10/28 (36)
Bastian et al. (1989)	14/18 (78)	13/16 (81)	13/18 (72)	18/18 (100)	ND
Driscoll et al. (1992a)	13/13 (100)	11/13 (85)	12/13 (92)	10/13 (77)	2/13 (15)
Wilson et al. (1993a)	42/44 (95)	40/44 (91)	33/44 (75)	44/44 (100)	23/44 (52)

* criteria for ascertainment
[1]immunodeficiency [2]dysmorphism [3]malformation
numbers represent the frequency with which a phenotypic feature was observed over the total number of patients ascertained
ND: not determined

noteworthy that these cardiac defects are rather uncommon in livebirths, and it has been estimated that 68% of interrupted aortic arch cases and 38% of truncus arteriosus also have DGS (Van Mierop and Kutsche, 1986).

Hypocalcemia can vary from complete absence of the parathyroids to transient hypocalcemia during the neonatal period. In many instances, a low calcium level has been reported with no particular symptoms in the patient (Finley et al., 1977; Conley et al., 1979; Wilson et al., 1991).

The thymus can be completely absent, hypoplastic or there can be immunodeficiencies in the presence of a thymus of normal appearance. The degree of thymic hypoplasia does not seem to be correlated with the severity of the immunological defects (Lagrue and Griscelli, 1984; Bastian et al., 1989). The immunological findings are characterized by a decrease in T8+ cells and regulatory imbalances between T and B cells (Durandy et al., 1986; Müller et al., 1989).

The facial dysmorphism most often includes lateral displacement of the inner canthi, short palpebral fissures, broad nasal bridge, short philtrum, small U-shaped mouth and low-set and posteriorly rotated ears. Association with other syndromes or with other malformations occurs quite frequently. These do not necessarily involve structures localized to the neck and chest (Conley et al., 1979). In no occasions were the 4 features present in all the cases of a particular series. As a general consensus, criterias necessary for a DGS diagnosis often included only 3 out of the 4 cardinal features (Table 1).

The variable spectrum of the DGS phenotype can also be illustrated by familial occurrences of this syndrome, where the clinical features of each affected members vary greatly. Many families have been reported where the clinical features displayed by the proband were much more severe than his/her affected parent, although an ascertainment bias cannot be excluded (Steele et al., 1972; Greenberg et al., 1984; Rohn et al., 1984; Keppen et al., 1988; Wilson et al., 1991). In addition, two occurrences of DGS in monozygotic twins have been reported (Miller et al., 1983; O. Ginsberg, personal communication). These two pairs of twins also showed differing phenotypes. As it was later demonstrated that the affected members in some of these families bear the same chromosomal rearrangement or deletion, the variable expressivity

observed in DGS could be explained by the influences of environmental factors or of stochastic events acting during embryonic development (Kurnit et al., 1987).

The frequency of DGS has been evaluated to 0.7% of 3469 consecutive post-mortem children studied (Conley et al., 1979) or 0.6% of children ascertained in pediatric cardiology and endocrinology units, giving an incidence for DGS of 5 in 100 000 children (Müller et al., 1988). But, as will be seen later, the increasing comprehension of the genetic defect involved in DGS unveiled a much higher prevalence than what was thought in these early studies.

1.2 Genetics

In most instances, DGS occurs sporadically, but there have been some reports of families segregating this syndrome (Steele et al., 1972; Raatikka et al., 1981; Atkin et al., 1982; Rohn et al., 1984; Keppen et al., 1988). Autosomal dominant, autosomal recessive and X-linked patterns of inheritance have all been proposed. The early observation of these familial cases has encouraged the search for a genetic cause of DGS.

The association of DGS with loss of the proximal part of chromosome 22 was first shown by the report of a large pedigree in which a t(20;22) (q11;q11) was segregating (de la Chapelle et al., 1981). A DGS phenotype was observed in 4 individuals, in 2 different branches of the family, in association with an unbalanced der(20) chromosome resulting in partial trisomy for chromosome 20 and monosomy for the 22pter-->q11 region. The phenotypes observed were not concordant with trisomy 20, but a link was made with two previous reports of monosomy 22 and features of DiGeorge syndrome (Rosenthal et al., 1972; DeCicco et al., 1973). However, these 2 cases of monosomy 22 could have in fact been unbalanced translocations. Some characteristics of DGS had also been found in another case of monosomy 22pter-->q11 through an unbalanced translocation (Back et al., 1980). Furthermore, the authors supposed that the phenotype was unlikely to be due to monosomy for the short arm of chromosome 22, since patients with r(22) or with Robertsonian

t(22;22) translocation do not have DGS. Nevertheless, this does not represent a definitive argument, since these 2 chromosomal rearrangements do not necessarily result in the loss of the entire short arm of chromosome 22. Therefore, the authors predicted that haploinsufficiency for a gene in 22q11 could cause DGS.

A number of reports has since confirmed this original observation (table 2). Kelley et al. (1982) reported on 3 unrelated patients with DGS who bore unbalanced translocations leading to monosomy 22pter->q11, with the remainder of chromosome 22 q-arm translocated to other autosomes. A partial monosomy for chromosome 22 was further identified in a mother and her DGS affected son (Greenberg et al., 1984). Two previous children from the same mother were displaying a DGS phenotype to varying degrees. The mother had only a mild immunodeficiency, facial dysmorphism and mental retardation.

An intriguing observation was that of a DGS patient with an apparently balanced t(2;22) (q14.1;q11.1) translocation (Augusseau et al., 1986). Her mother, brother and a maternal aunt bore the same translocation. The mother was considered normal at that time and the maternal aunt has had her 4th pregnancy terminated because of fetal congenital heart disease and other anomalies.

Eight other cases of monosomy 22pter-->q11 due to aberrant segregation of translocation products have been reported in the literature (table 2). One last case of inheritance of a t(18;22) (q12;p11.2)pat translocation in a patient with DGS and a deletion restricted to the short arm of chromosome 22 has been observed (Bowen et al., 1986). It was hypothesized that a subsequent undetected deletion could have occurred in the proximal long arm of chromosome 22 as a result of non-homologous recombination during paternal meiosis. This situation was said to be reminiscent of 2 Prader Willi syndrome cases associated with apparently balanced t(15;15) Robertsonian translocations with 2 centromeres.

Greenberg et al. (1988a) were the first to report an interstitial deletion of 22q11.21-q11.23 in a DGS patient. A similar finding was later reported by Mascarello et al. (1989) and confirmed in 13 other DGS cases (Wilson et al., 1992; Desmaze et al., 1993b; Francke et al.,

Table 2

Cytogenetic findings in DiGeorge syndrome

REFERENCE	CYTOGENETIC ABNORMALITIES
Chromosome 22 abnormalities	
Rosenthal et al., 1972	45,XY,-22
DeCicco et al., 1973	45,XX,-22
Back et al., 1980	45,XX,-11,-22,+der(11),t(11;22)(p15;q11)
de la Chapelle et al., 1981	45,-20,-22,+der(20),t(20;22)(q11;q11) 3 cases
Kelley et al., 1982	45,XX,-10,-22,+der(10),t(10;22)(q26;q11)
	45,XX,-3,-22,+der(3),t(3;22)(q29;q11)
	46,XY,-22,+der(20),t(20;22)(q11;q11)
Greenberg et al., 1984	45,-4,-22,+der(4),t(4;22)(q35;q11)mat
Pong et al., 1985	46,XY/46,XY,del(22)(pter->q11)
Augusseau et al., 1986	46,XX,t(2;22)(q14.1;q11.1)mat
Bowen et al., 1986	46,XY,-22,+der(18),t(18;22)(q12.2;p11.2)pat
Kaplan et al., 1987	45,t(1;22)
Faed et al., 1987	45,XX,-3,-22,+der(3),t(3;22)(p25;q11)
Schwanitz and Zerres, 1987	45,X,t(X;22)(q21;q11)
Greenberg et al., 1988a	45,XX,-20,-22,+der(20),t(20;22)(q13;q11)
	46,XY,del(22)(q11.21;q11.23)
Mascarello et al., 1989	46,XX,del(22)(q11.21;q11.23)
Pinto et al., 1989	45,X,-X,-22,+der(X),t(X;22)(p21.3;q11.2)
Dallapiccola et al., 1989	45,t(X;22)((q28;q11.1)
Pivnick et al., 1990	46,XX,-22,der(9),t(9;22)(q22;q11.2)mat
El-Fouly et al., 1991	46,XX,-22,+der(9),t(9;22)(q21.13;q12.1)mat
Lupski et al., 1991	45,X,-Y,-22,+der(Y),t(Y;22)(p11;q11.2)pat
Wilson et al., 1992a	46,del(22)(q11.21;q11.23) 9cases
Driscoll et al., 1992a	46,del(22)(q11.21;q11.23) 1 case
Desmaze et al., 1993b	46,del(22)(q11.21;q11.23) 2 cases
Francke et al., 1994	46,del(22)(q11.21;q11.23) 2 cases

REFERENCE	CYTOGENETIC ABNORMALITIES
Chromosome 10 abnormalities	
Bridgman and Butler, 1980	46,XX,-10,+der(10),t(10;14)(p14;q22)
Gencik, 1983	46,XY,del(10)(p13)
Hervé et al., 1984	46,XY,del(10)(p13)
Koenig et al., 1985	46,XY,del(10)(p13)
Greenberg et al., 1986	46,XX,del(10)(p13)
Debevec and Brezigar, 1986	del(10)(p13)
Monaco et al., 1991	46,XY,del(10)(p13)
Lai et al., 1992	46,XX,-10,+der(10),t(5;10)(q35.2;p13)mat
Obregon et al., 1992	46,XY,del(10)(p11;p13)
Goodship et al., 1994	46,XX,-10,+der(10)t(3;10)(p23;p13)mat
Others	
Van den Berghe et al., 1973	46,XY,-12,+der(1),t(1q;12q)
Townes and White, 1978	46,X,dup(8)(q22->qter)
Taylor and Josifek, 1981	46,XX,del(5)(p13)
Schinzel, 1984	trisomy 18
Greenberg et al., 1988b	46,XX,del(17)(p13)
Van Essen et al., 1993	46,XX,i(18q)
Lindgren et al., 1994	46,XX,dirdup(9),(q21.12->q22.1)

1994). The patients, however, did not display a milder phenotype because of the smaller size of their deletions compared to unbalanced translocation carriers. Therefore, all these observations seemed to document convincingly a causal relationship between 22q11.2 deletions and DGS.

Isolation of molecular probes from the proximal part of chromosome 22 have permitted refining of the shortest region of overlap in DGS patients (Carey et al., 1990; Scambler et al., 1991a; Fibison et al., 1990; Driscoll et al., 1992a). Analysis of patients with normal chromosome complements using these probes has

demonstrated that over 90% of patients affected with DGS have 22q11.2 deletions (Driscoll et al., 1992a; Desmaze et al., 1993b; Carey et al., 1992; Goldmuntz et al., 1993).

Nevertheless, a number of other cytogenetic abnormalities has been noted to be associated with DGS; namely monosomy 10p13 and 17p13 (table 2). This is taken as evidence of causal heterogeneity for this developmental defect. A total of 9 DGS patients have been found to carry 10p13 deletions. The cardiac defect did not involve the great vessels of the heart in one case (Obregon et al., 1992) and was absent in 2 (Greenberg et al., 1986; Monaco et al., 1991). Hypocalcemia was manifest in all patients and thymus aplasia or immunodeficiencies in all but one case (Goodship et al., 1994). Nevertheless, dysmorphic facial features were noted to be distinct from those seen in DGS resulting from chromosome 22 deletions (Goodship et al., 1994). Therefore, there appears to be another possible locus for a DGS-like pathology in the 10p13 chromosomal region, but no candidate gene has been mapped to this chromosomal region.

1.3 Pathogenesis and Etiology

In addition to the many genetic defects outlined in the previous section, the DGS phenotype has been observed after a number of teratogenic disruptions in humans and animals (reviewed in Lammer and Opitz, 1986). Furthermore, some animal models showing the phenotypic features of DGS have been described. Because of this causal heterogeneity, it was suggested that DGS should be really called the "DiGeorge anomaly" (DGA) or the "DiGeorge malformation complex" (Carey, 1980; Lammer and Opitz, 1986).

Five infants with DiGeorge anomaly have been born to mothers with diabetes (Black et al., 1975; Gosseye et al., 1982; Wilson, T.A. et al., 1993; Puno-Cocuzza et al., 1994). In addition to the usual manifestations of the DGA, all children had unilateral renal atresia and vertebral defects, as well as various other malformations not commonly associated with the DiGeorge malformation pattern. One of the mothers had phenotypic features found in the Velo-Cardio-Facial syndrome (VCFS). Three patients, and the VCFS-like mother were

examined for the presence of deletions in the 22q11.2 region and were all found normal (Wilson, T.A. et al., 1993; Puno-Cocuzza et al., 1994). Therefore, maternal diabetes-induced teratogenesis is likely to be a possible etiology in a very small proportion of DGS cases.

The DGA has been observed in children of alcoholic mothers (Ammann et al., 1982). Furthermore, an increased susceptibility to infections due to decreased T-cell immunity was associated with the fetal alcohol syndrome (Johnson et al., 1981). Similar immunodeficiencies could be produced by exposing rats in utero to alcohol throughout gestation (Monjann and Mandell, 1980). Sulik et al. (1986) could induce a pattern of malformations very similar to that observed in DGA by acute maternal exposure to alcohol in mice, at day 8 of gestation. Ethanol administration at a time when cranial neural crest cells migrate into the branchial arches, resulted in decreased mesenchymal contribution of neural crest origin in the arches and general size reduction of branchial arches, cerebral hemisphere and nasal prominences. The consistency of the findings in human case reports and the existence of experimental models give credence to a causal relationship between fetal alcohol exposure and the DGS phenotype.

Exposure to vitamin A or one of its analog (isotretinoin) *in utero*, produces malformations involving craniofacial, cardiac, thymic and central nervous system structures in about 20% of human infants (Lammer et al., 1985). The type of cardiac malformations produced closely resembles those found in the DGA (Lammer and Opitz, 1986). Administration of retinoids during gestation in experimental animals induces the same type of malformations (Poswillo, 1975; Davis and Sadler, 1981; Johnston et al., 1985). It was hypothesized that the craniofacial, cardiac and thymic defects observed after exposure to isotretinoin in human infants, was due to an alteration in the normal function or interactive influence of cranial neural crest cells (Lammer et al., 1985). This hypothesis was supported by evidence that mouse fetuses exposed to retinoic acid and showing the same type of malformations than human infants, had defects specific of cephalic neural crest cell populations (i.e. dysmorphic neural crest cells,

defective migration patterns) (Johnston et al., 1985; Goulding and Pratt, 1986).

DGA can also be caused by exposure of rat fetuses to a bis-dichloroacetylamine (WIN 18 446), a fat-soluble zinc chelating agent, during day 9 to 12 of gestation (Oster et al., 1983; Okishima et al., 1984) and it has been postulated that zinc metabolism could play a role in the pathogenesis of DGS.

A mouse model for DGS has also been produced by targeted disruption of both copies of the homeobox containing gene *hox-1.5* in pluripotent mouse embryonic stem cells (Chisaka and Capecchi, 1991). The homozygote animals had no thymus nor parathyroids, decreased thyroid tissue, face and throat malformations and cardiac defects involving the great vessels. Mice heterozygous for a null allele of *hox-1.5* appeared normal. The phenotypic features of mice with both of their *hox-1.5* alleles inactivated, and of DGS patients are remarkably similar. However, the human homolog to murine *hox-1.5* maps to chromosome 7p13 and DGS in humans is a dominant genetic defect. It is possible that both genes act in the same developmental pathway.

Given the diverse teratogenic or genetic factors which can result in a DGS phenotype, this syndrome is considered a developmental field defect (Lammer and Opitz, 1986). A developmental field is a group of embryonic cells that develop in a coordinated manner and that react in the same way to different environmental insults or genetic causes (Opitz, 1985).

The structures involved in the DGA are all derived from the 3rd and 4th pharyngeal pouches. It has been shown that the neural crest makes important mesenchymal contributions to the derivatives of the pharyngeal pouches. Using the quail-chick chimera experimental model, Lelièvre and LeDouarin (1975) showed that the cephalic neural crest cells contribute mesenchymal tissue to the bones and connective tissues of the face, as well as to the musculoconnective tissue wall of the great vessels of the heart. The cephalic neural crest cells have also been shown to migrate into the conotruncal region of the heart and participate in the process of aorticopulmonary septation (Kirby et al., 1983). Extirpation of the neural crest between

1st to 5th somite in chicken embryos, resulted in thymus, parathyroids and sometimes thyroids of markedly reduced size, if not completely absent (Bockman and Kirby, 1984).

The mechanism by which alcohol and retinoic acid exposure during embryogenesis can result in a DGS phenotype also appears to be through an altered mesenchymal contribution from the neural crest (Sulik et al., 1986; Johnston et al., 1985; Goulding and Pratt, 1986). In particular, ethanol may disorder membrane structure and thus be deleterious to rapidly proliferating or migrating cells (Sulik et al., 1986). Zinc apparently stabilizes cell membranes and has been shown to decrease the teratogenic effects of ethanol (Tanaka et al., 1982). Finally, it has been suggested that Hox genes could provide positional information to the branchial arches and pharyngeal pouches, by "imprinting" neural crest cells before their migration, with a distinct Hox code specific of a particular rhombomere (Hunt et al., 1991a). Altered expression of the gene *Krox 20*, a zinc finger gene which is thought to be involved in the initial segmentation process that establishes rhombomeres before the homeobox genes come into play, could represent an alternative way through which zinc deficiency can generate a DGS phenotype (Hunt et al., 1991b).

Therefore, the developmental field in DiGeorge anomaly is the population of cephalic neural crest cells that migrates into the pharyngeal pouches and the defect may be attributed to failure to attain a critical number of neural crest cells (Kirby and Waldo, 1990). Supporting this view is the demonstration that patients with the DGA have significantly reduced number of neural crest-derived thyrocalcitonin immunoreactive cells in their thyroid gland compared to controls (Burke et al., 1987; Palacios et al., 1993). This decreased number of cephalic neural crest cells in the affected structures of DGS could be caused by a failure of proper migration, an excessive cell death or a defective interaction with the pharyngeal pouches. It is therefore possible that all the genetic defects or teratogenic disruptions involved in DGS, act on this developmental field.

2. Related syndromes associated with chromosome 22 deletions: The CATCH 22 group of syndromes

Because of the wide clinical variability observed in DGS and of the clinical overlap between DGS and other syndromes it was hypothesized that chromosome 22q11.2 deletions may be etiologically related to these syndromes. This hypothesis turned out to be true. Chromosome 22q11.2 deletions have been uncovered in several syndromes and malformations such as the Velo-Cardio-Facial syndrome (VCFS), the Conotruncal-Anomaly-Face syndrome (CTAF) and isolated conotruncal cardiac defects. All these syndromes have been grouped under the acronym CATCH 22 (Cardiac defect, Abnormal facies, Thymus hypoplasia, Cleft palate, Hypocalcemia and chromosome 22 deletion) to denote the great phenotypic variability associated with 22q11.2 deletions (Wilson et al., 1993a).

2.1 The Velo-Cardio-Facial syndrome (Shprintzen syndrome)

The Velo-Cardio-Facial syndrome (VCFS) was first described in 1978 by Shprintzen et al. It is a disorder with multiple malformations including cleft palate, conotruncal heart defect and a typical facial dysmorphism. Over 100 cases have been described. Clinically, the great majority of patients have learning disabilities and a submucous or overt cleft palate (it is the most common syndrome associated with clefting) (Goldberg et al., 1993). Typically, the facial features include prominent nose with broad nasal root, narrow palpebral fissures, malar flatness and retruded chin (Shprintzen et al., 1981). Microcephaly occurs in 40% of cases and cardiovascular defects (mainly ventricular septal defect) in 80% (Goldberg et al., 1993). The analogous phenotype of DGS and VCFS was first recognized by Young et al. (1980). DGS occurs as a component manifestation of the syndrome in at least 10% of cases (Goldberg et al., 1985), and in fact many patients first published as DGS were retrospectively diagnosed as VCFS (Stevens et al., 1990).

Before the advent of molecular studies, there had been 5 VCFS families reported where the affected parent was the mother and one case of father to son transmission of VCFS suggesting an autosomal dominant mode of inheritance, but the variable expressivity of all features (especially within families) was already recognized (Strong, 1968; Shprintzen et al., 1981; Williams et al., 1985).

The clinical overlap between DGS and VCFS prompted the search for 22q11.2 deletions in VCFS patients. Molecular deletions were discovered in 25 out of 26 patients studied (Scambler et al., 1992; Driscoll et al., 1992b; Kelly et al., 1993). In addition, the VCFS deleted region appeared to be the same as the DGS deletion, at least at the resolution provided by the markers used. It has been proposed that the 2 syndromes are different manifestations of haploinsufficiency for the same gene(s) (Scambler et al., 1992; Driscoll et al., 1992b).

There has been much discussion as to whether chromosomal region 22q11.2 is the DGS region or the VCFS region (Stevens et al., 1990; Motzkin et al., 1993; Shprintzen et al., 1994). It is true that many cases of DGS have been diagnosed retrospectively as VCFS. In addition, 4 families have been reported in which DGS was occurring as a feature of VCFS (Rohn et al., 1984; Keppen et al., 1988; Stevens et al., 1990). Two of these families have been first ascertained as DGS families (Rohn et al., 1984; Keppen et al., 1988). Second, of 60 patients reported with the VCFS, 60% were noted to have an excessive number of infections, 90% to have decreased lymphoid tissue and 20% to have had an episode of neonatal hypocalcemia, all features associated with DGS (Goldberg et al., 1985; 1993). Third, facial dysmorphism was not included as a feature of DGS in the original reports (DiGeorge, 1965; 1968; Conley et al., 1979), although it had been noted. Furthermore, there were 3 patients with palatal anomalies in the Conley et al. (1979) study. Published photographs of some patients reported to have DGS demonstrate facial features consistent with VCFS (Krestchmer et al., 1968; Freedom et al., 1972; Shimisu et al., 1984; Radford et al., 1988). In this view, DGS is considered as a developmental field defect that is causally heterogeneous and that can occur as a component of several

syndromes including VCFS (Stevens et al., 1990). Familial transmission of DGS would actually represent VCFS with DGS as a submanifestation (Stevens et al., 1990; Goldberg et al., 1993). According to Shprintzen (1994), VCFS has only one etiology: chromosome 22q11.2 deletions. The diagnostic label would then depends on the area of interest of the clinician (cardiology, immunology or dysmorphology).

Nevertheless, there is an increasing body of evidence to suggest that these 2 conditions are both ends of the range of manifestations of one genetic defect. Like DGS, there are several observations of typical VCFS cases displaying no 22q11.2 deletions (Driscoll et al., 1993; Siegel-Bartelt et al., 1994; Ravnan et al., 1994). However, these cases could be due to deletions that are undetectable with the current markers, or these cases may have point mutations within the major gene causing the syndrome. The VCFS, like DGS, is very variable in expression. There has been 2 examples of familial VCFS with discordance for palatal anomalies and cardiac defects (McLean et al., 1993; Holder et al., 1993) and 2 cases of VCFS with only the typical facial dysmorphism whose clinical manifestation was hypoparathyroidism. All these cases were associated with 22q11.2 deletions (Scirè et al., 1994). It is therefore more likely that most cases of VCFS or DGS arises as a result of haploinsufficiency for one gene located in 22q11.2 and that the resulting phenotype depends on chance (Kurnit et al., 1987), on the genetic background of the individual or on the clinical criteria for ascertainment of the patients. The remaining VCFS cases showing no chromosome 22 deletions could be the result of a teratogen exposure during pregnancy or of another genetic cause.

2.2 The Conotruncal Anomaly Face syndrome

The Conotruncal Anomaly Face syndrome (CTAF) associates cardiac outflow tract defects, a characteristic facies (hypertelorism, narrow palpebral fissures, lateral displacement of the inner canthi, flat nasal bridge), a nasal voice and minor ear anomalies (Kinouchi et

al., 1976). The authors subsequently noted association of CTAF with immunodeficits and facial features consistent with DGS, and clinical overlap with VCFS (Takao et al., 1980; Shimizu et al., 1984). Five patients with this syndrome were investigated by high resolution chromosome analysis and fluorescence *in situ* hybridization (FISH) with a cosmid probe from the DGS deleted region and a deletion was observed in all of them (Burn et al., 1993).

Many authors have suggested that VCFS and CTAF are the same entity or that at least, a subset of CTAF patients are really VCFS (Gorlin et al., 1990; Stevens et al., 1990; Golberg et al., 1993), again suggesting that the belief that DGS, VCFS and CTAF are distinct syndromes depends ultimately on the clinical area of expertise of the ascertaining researcher.

2.3 Isolated conotruncal cardiopathies

The finding of 22q11.2 deletions in 2 siblings of a DGS patient which were only affected with a congenital heart defect (ventricular septal defect in one, coarctation of the aorta in the other) prompted the investigation of families with recurrence of congenital heart disease (Wilson et al., 1991). The idea was not so far-fetched since it has been observed that recurrence risk for offspring of parents who have congenital heart disease is substantially higher, particularly if the affected parent is the mother (Nora and Nora, 1987). Nine families were investigated and 5 of them were found to segregate a 22q11.2 deletion (Wilson et al., 1992b). However, in 2 of these families, another feature of DGS was also observed. Also of note, was that the transmission of the deletion was associated with an increase in the severity of the cardiac defect and no deletions were found in families where sibling pairs had identical heart defect. Goldmuntz et al. (1993) reported on another case of familial congenital heart disease associated with a chromosome 22 deletion.

Similar studies were performed in cases of isolated conotruncal heart defects. Two deleted subjects were identified out of 40 cases of tetralogy of Fallot examined (5%) (Wilson et al., 1992b). Four

individuals were found deleted out of 14 studied with isolated conotruncal cardiopathies (20%), but subtle dysmorphic features, although not characteristic of a syndrome, were also reported in these patients (Goldmuntz et al., 1993). The same group later reported to have found 13 deleted cases out of 20 patients (65%) with a conotruncal cardiac anomaly. The deleted patients were then evaluated for palatal function and speech and 7 were noted to have velopharyngeal incompetence (McDonald-McGinn et al., 1994). It is likely that in this study, subtle features of the CATCH 22 syndrome went unnoticed, which would explain this high frequency of 22q11.2 deletions.

In a recent study, 14 patients with a cyanotic congenital heart lesion were examined by dysmorphologists and 6 of them (all affected with pulmonary atresia and ventricular septal defect) could be grouped together on the basis of craniofacial appearance (Seaver et al., 1994). Four of these underwent molecular screening for 22q11.2 deletions and were, in fact, found to bear a deletion on their maternally inherited chromosomes.

In conclusion, the frequency with which isolated conotruncal cardiopathies are associated with chromosome 22 deletions seems to vary over a wide range. Further investigations are needed to estimate a precise prevalence, notably prospective studies with very strict inclusion criteria.

2.4 Other syndromes

The DGS clinical features have also been found as part of the CHARGE association (Coloboma of the eye, Heart defect, choanal Atresia, Retarded growth/development, Genital hypoplasia and Ear anomalies) in at least 9 instances (Pagon et al., 1981; Siebert et al., 1985; Clementi et al., 1991; Emanuel et al., 1992), where one case was the carrier of an unbalanced t(3;22) (p25.1;q11.1)mat translocation, and two had chromosome 22 interstitial deletion revealed by molecular analysis. It is noteworthy that the CHARGE association, just like DGS/VCFS has also been found associated with

Kallmann syndrome, with the Pierre-Robin sequence (micrognathia and cleft palate) and with isolated cleft palate or bifid uvula (Shen et al., 1979; Pagon et al., 1981; Shprintzen et al., 1981). All of these syndromes or malformation patterns involve developmental defects of the anterior part of the body and in one (the Kallmann syndrome) altered neuronal cell migration has been suggested.

Noonan's syndrome (a genetically heterogeneous condition characterized by congenital heart defect, short stature, chest deformities, short webbed neck and facial dysmorphism) has been seen in association with DGS in 2 instances. One case of DGS with 22q11.2 deletion has been reported, that in addition, presented with Noonan's syndrome (Wilson et al., 1993b), whereas another case of "Noonan-like appearance" daughter born from a VCFS mother was reported, with 22q11.2 deletion in both patients (Piussan et al., 1994).

Finally 2 cases of VCFS and 2 of DGS, all carrying chromosome 22 deletions were found to be associated with meningomyelocele, a defect of neural tube closure (Palacios et al., 1993; Nickel et al., 1994). Neural tube defects and congenital heart diseases are associated in 5% of cases. Involvement of neural crest cells has been proposed, especially since retinoic acid and alcohol both can cause neural tube defects, congenital heart diseases, cleft palate and as mentioned previously, a pattern of defects very similar to DGS.

2.5 Conclusion

We have seen that submicroscopic deletions within 22q11.2 are associated with a variety of phenotypic defects, most importantly with DGS, VCFS and isolated conotruncal cardiac defects, and generally with phenotypic defects having in common facial dysmorphism, conotruncal heart defect and development of the anterior structure of the body. Haploinsufficiency for 22q11.2 has been estimated to be the cause of at least 5% of congenital heart defects with a minimum prevalence of 1/4000 livebirths (Wilson et al., 1994). It thus appears that there is/are very important gene(s)

on chromosome 22 playing a part in embryological development of the 3rd and 4th pharyngeal pouches and especially of conotruncal development of the heart.

3. Physical mapping of chromosomal band 22q11.2

Chromosomal band 22q11.2 is the site of a striking number of constitutional or acquired rearrangements, despite its relatively small size (5-10 Mb). The cancer-related acquired rearrangements of chronic myelocytic leukemia, [t(9;22)], acute lymphocytic leukemia, [t(9;22)], Burkitt lymphoma, [t(8;22)] and Ewing sarcoma, [t(11;22)], all involve this chromosomal band. In addition, the recurrent balanced translocation t(11;22) (q23;q11) associated with the supernumerary der(22) syndrome represents the most common non-Robertsonian translocation in humans. Finally, other constitutional rearrangements involving the 22q11.2 region include the cat eye syndrome (which is characterized by trisomy or tetrasomy for the proximal part of 22q11.2) and DGS resulting from monosomy for 22q11.2 (reviewed in Kaplan et al., 1987). The construction of a detailed physical map of this genomic region has greatly increased the understanding of the pathogenesis of these diseases.

3.1 DiGeorge syndrome: commonly deleted and critical regions

Molecular genetic methods have been used to isolate new probes or map loci more precisely within this chromosomal band. Probes from a number of sources have been isolated, including chromosome 22-enriched flow-sorted libraries, microdissection libraries, Not I linking clone libraries and genomic libraries prepared from somatic hybrids containing whole or part of chromosome 22 (McDermid et al., 1989; Budarf et al., 1991; Carey et al., 1990; Sharkey et al., 1992; Sanson et al., 1992; Lamour et al., 1993; Kurahashi et al., 1994). This work has helped to saturate the region

with new probes that could be used for deletion searches in DGS by RFLP or dosage, and to construct a long range pulsed-field gel electrophoresis map of the region.

These new or already existing probes were then precisely mapped using panels of chromosome 22 cell hybrids and were used to search for deletions in DGS patients with normal karyotypes and to delimit the region of haploinsufficiency in DGS cases bearing interstitial deletions or unbalanced translocations (Fibison et al., 1990; Carey et al., 1990; Scambler et al., 1991a). One finding that emerged from these studies was that DGS was associated at a high frequency with deletions within 22q11.2 that are too small to be detectable by karyotype analysis (Scambler et al., 1991a; Driscoll et al., 1992a; Carey et al., 1992).

The next step was to order the loci found deleted in DGS in order to delineate a shortest region of overlap, i.e. the minimal critical region which when deleted will give rise to the disease. A variety of tools and approaches were used toward this end by the different groups working on the developmental defects associated with 22q11.2 deletions. Chromosome 22 hybrid panels made from constitutional, acquired or radiation reduced rearrangements, were used to map grossly the interesting probes. Linkage analysis or two-color FISH was used to order them. One interesting and invaluable approach has been to take advantage of the chromosomal rearrangements carried by some DGS patients to map loci on one derivative or the other. Finally, distances were estimated by two-color FISH or PFGE.

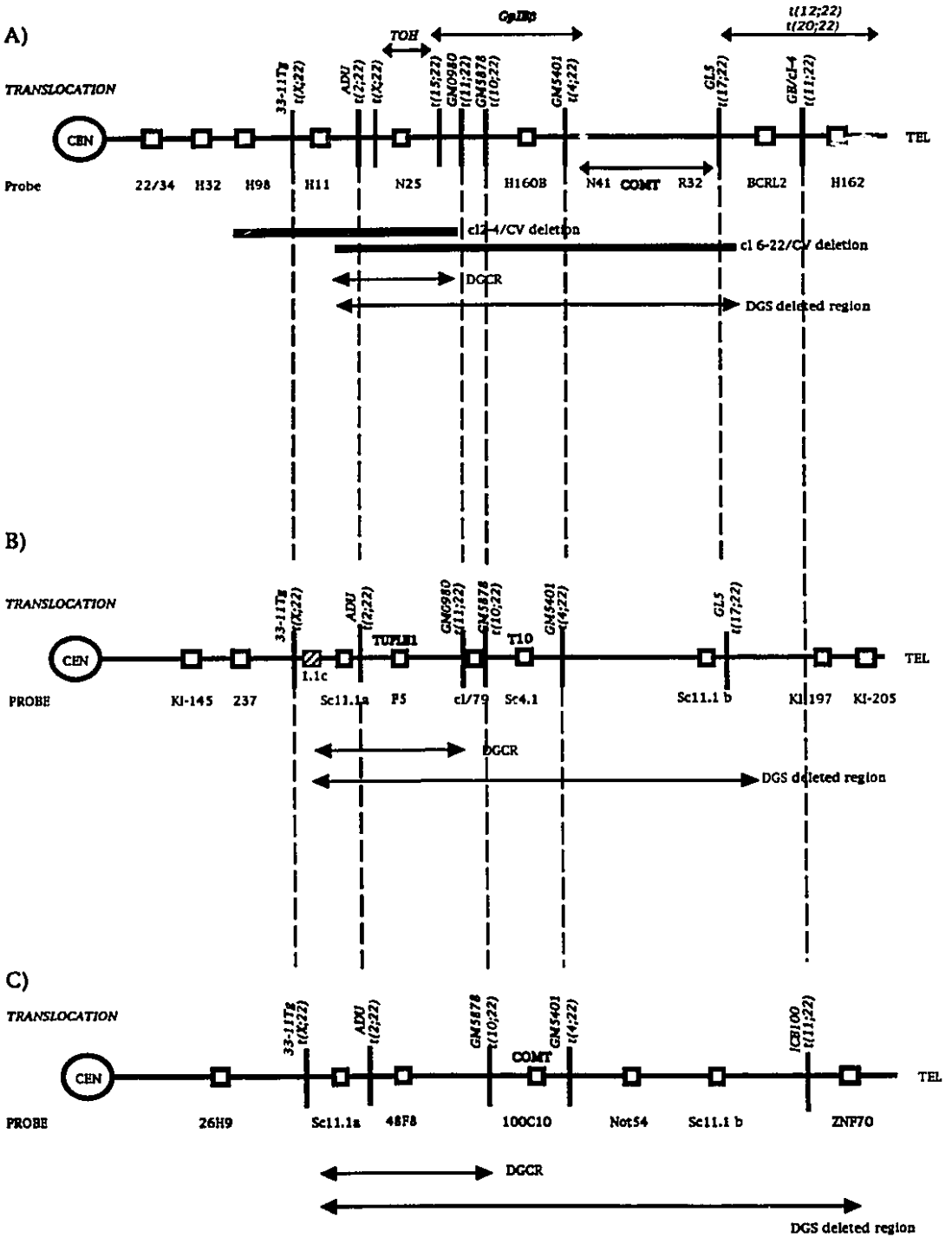
Figure 1 shows the DGS deleted and critical regions as defined by the 3 research groups (B.S. Emanuel, Hosp. for Sick Children, Philadelphia; P.J. Scambler, Inst. of Child Health, London; A. Aurias, Curie Inst., Paris) working on the chromosome 22 microdeletion syndromes. Panel A represents the DGS region from Dr. Emanuel's group. DGS patients had differing extents of deletions, but 3 probes were always found deleted: N25 (D22S75), H160B (D22S66) and R32 (D22S259) (Driscoll et al., 1992a). Together, these probes cover over 750 kb of genomic DNA and are deleted in 30 of 33 DGS patients studied (Budarf et al., 1992; Emanuel et al., 1993), thereby defining

the commonly deleted region. A PFGE map of this region later estimated its size more precisely to 1.6 Mb and revealed that many Not I fragments were smaller than 50 kb, suggesting that the region has many CpG islands (Budarf et al., 1994a). Recently, new balanced (ADU, TOH) and unbalanced [t(X;22), t(15;22), GM00980 t(11;22), t(12;22), t(20;22)] translocation, as well as an atypical deletion associated with DGS (cl2-4/CV) have been placed in this map (Emanuel et al., 1993; Budarf et al., 1994a; Li et al., 1994), therefore delimiting a critical region flanked by pH11 (D22S36) and the t(X;22) breakpoint. As the der(X) was inactivated in both affected sibs, the possibility was raised that spreading of inactivation on the translocated 22-derived segment could silence gene(s) distal to the breakpoint (Li et al., 1994). Thus, a more conservative estimate of the critical region puts it between pH11 (D22S36) and the t(15;22) translocation breakpoint, which makes the 2 balanced translocation breakpoints (ADU and TOH) fall within the 200 kb DGCR determined by this group (B.S. Emanuel, personal communication). Seventy percent of the commonly deleted region has been contiged into cosmids and YACs.

Figure 1B shows the physical map of the DGS region of Dr. Scambler's group. The deleted region extends in some patients from probe Sc11.1a to KI-197 (D22S111) (Scambler et al., 1991a). Probe Sc11.1 recognizes 2 loci that are 1 to 2 Mb apart (Sc11.1 a and b) and that are both found deleted in approximately 90% of patients studied, thereby defining the extent of the commonly deleted region (Scambler, 1993). Since probe KI-197 has been rarely used for deletion screening, it is not known if it is part of the commonly deleted region or not. Using two-color FISH and translocation breakpoint mapping, a molecular cytogenetic physical map of the region was established. The distance between Sc11.1a and F5 was estimated to 300 kb minimally and these 2 loci have always been found deleted in over 100 patients studied (Lindsay et al., 1993; Scambler, 1993). Therefore, the boundaries of the critical region were defined as the proximal limit of the commonly deleted region down to the GM00980 t(11;22) breakpoint (Lindsay et al., 1993; Halford et al., 1993a). The use of FISH to map cosmid loci with respect to

Figure 1

Comparison of the limits of the DGS critical and commonly deleted regions between 3 research groups: A) B.S. Emanuel, Philadelphia; B) P.J. Scambler, London; C) A. Aurias, Paris. Vertical lines indicate translocation breakpoints from hybrids or DGS patients; squares designate loci. Black rectangles in panel A show the extent of 2 interstitial deletions; the stipled rectangle in panel B indicates a locus that is part of one of the low-copy-repeat families identified by this group. The DGS commonly deleted and critical regions are shown by double-headed arrows under the schematic chromosome 22. When no information is available on the precise localization of a particular probe, breakpoint or gene, the maximum area in which it can be found is delimited by arrows.


A)
TRANSLOCATION
CEN
TEL
Probe
22/34
H32
H98
H11
N25
H160B
N41
COMT
R32
BCRL2
H162
TOH
GpIIb
t(12;22)
t(20;22)
33-11Tg t(X;22)
ADU t(2;22) t(X;22)
t(15;22)
GM0980 t(11;22)
GM5878 t(10;22)
GM5401 t(4;22)
GL5 t(17;22)
GB/cl-4 t(11;22)
cl2-4/CV deletion
cl 6-22/CV deletion
DGCR
DGS deleted region
B)
TRANSLOCATION
CEN
TEL
PROBE
KI-145
237
Sc11.1a
1.1c
F5
cl/79
Sc4.1
Sc11.1 b
KI-197
KI-205
TUPLE1
T10
DGCR
DGS deleted region
C)
TRANSLOCATION
CEN
TEL
PROBE
26H9
Sc11.1a
48F8
100C10
Not54
Sc11.1 b
ZNF70
COMT
IGB100 t(11;22)
DGCR
DGS deleted region

translocation breakpoint has enabled this group to identify several low-copy-repeat families that flank the DGS commonly deleted region. Besides Sc11.1, several markers detected repeated sequences (Carey et al., 1990), and all the loci recognized by these probes appeared to be conserved in several species (Halford et al., 1993c). Analysis of primates' DNA with some of these probes revealed that the repeat families they recognize have arisen relatively recently in mammalian evolution (between 55 to 11 million years ago) (Halford et al., 1993c). The presence of these repeats at the DGS deletion borders have been hypothesized to render the region meiotically unstable and to lead to frequent non-homologous recombination events (Scambler et al., 1991b).

The group of Dr. A. Aurias with which I have been collaborating have their physical map depicted in figure 1C. However, the data presented are rather outdated, since most of the work I have been doing in the last 2 years was to refine this map. Nevertheless, this first version has laid the ground work for the more recent version of the DGS physical map which is presented in Chapter 4. This map was devised using FISH on DGS translocation carriers and two-color FISH. The commonly deleted region extended from D22S9 to ZNF70 and the critical region from distal to the t(X;22) 33-11TG hybrid breakpoint to the GM05878 breakpoint (Desmaze et al., 1993a).

Work on the DGS region has lead to several conclusions. Firstly, that DGS is often associated with submicroscopic deletions that are considerably larger than the critical region. The presence of the repetitive sequence families on either side of the deletion could explain why that is so. Secondly, there does not appear to be any correlation between the severity of the phenotypic features in the DGS patients and the extent of the deletions: patients with no detectable deletions or with deletion detected with only one probe, can be as severely affected as patients that are hemizygous for the whole commonly deleted regions (Desmaze et al., 1993a; Scambler, 1993; Budarf et al., 1994a). This also applies for deletions detected in patients affected with VCFS or conotruncal heart disease (Kelly et al., 1993; Driscoll et al., 1992b, Wilson et al., 1992b). Most of these patients are deleted for the same chromosome 22q11.2 region as the

DGS affected patients. Thirdly, the size and extent of the critical and commonly deleted regions seem to be in concordance between the different research groups.

3.2 Genes isolated from the DGS deleted region

In order to identify the genes involved in the pathogenesis of DGS and related syndromes, a number of approaches have been used. Some groups have adopted a random approach, by constructing genomic libraries from somatic hybrids containing whole (Kurahashi et al., 1994) or part of (Lamour et al., 1993) chromosome 22. Some clones were found to be deleted in DGS patients and to cross-hybridize to rodent sequences or to contain CpG islands. Other groups are using large scale approaches and are building transcription maps of the whole DGS region (Gong et al., 1994; Sirotkin et al., 1994). These recent endeavours have not yet yielded a good candidate gene for DGS, but should prove valuable toward this end, especially if more than one gene, or a gene family is found to be responsible for these syndromes.

A third approach has been to isolate coding sequences from single-copy probes mapping within the DGS deleted region (Aubry et al., 1993; Budarf et al., 1992; Driscoll et al., 1992a; Wadey et al., 1993; Halford et al., 1993a, b) or to map already cloned genes within the DGS region (Grossman et al., 1992; Patracchini et al., 1992). I will now review the data available on each of these genes, starting from the distal part of the DGS deleted region, up to the proximal part.

ZNF74/cos40 (fig. 1C): ZNF74 is a zinc finger-containing gene that has been isolated by homology screening of a chromosome 22-enriched cosmid library (Aubry et al., 1992). It was found deleted in 23 out of 24 DGS patients tested (the patient with no ZNF74 deletion did not have a deletion for any of the other 22q11.2 probes tested) and mapped in the distal part of the DGS deleted region, with direction of transcription from centromere to telomere (Aubry et al., 1993). It contains zinc finger motifs of the Cys_2-His_2 Kruppel/TFIIIA family for which some members are known to be transcriptional

regulators. Involvement in the DGS pathogenesis was postulated on the basis of its expression in fetal human and mouse tissues, its function as a probable transcriptional regulator and the existence of a DGS animal model by administration of a zinc chelator (see section 1.3).

N41 (fig. 1A): It is a chromosome 22 specific Not I linking clone (McDermid et al., 1989) which potentially contains a CpG island. One cDNA clone was obtained by screening a human fetal liver library and it detects a 4.4 kb transcript in several human tissues and in 11.5 day mouse embryos (Budarf et al., 1992). Sequencing data revealed that N41 is a novel gene which shares a high degree of homology with the corresponding mouse cDNA, but no information on database searches were provided (Emanuel et al., 1993).

Catechol-O-methyl-transferase (fig. 1A,C): The catechol-O-methyl-transferase (COMT) enzyme catalyzes the metabolism of catecholamine such as norepinephrine, epinephrine and dopamine to inactive O-methyl esters. This gene has been recently mapped to 22q11.2 (Grossman et al., 1993; Winqvist et al., 1993). High- and low-activity alleles for the soluble COMT have been demonstrated in human populations (MIM 21273, 1983), and low COMT activity has been reported in women with primary affective disorder (Dunham et al., 1992). The recent observation that at least 10% of teenagers or adults VCFS patients develop psychiatric disorders (Shprintzen et al., 1992) has put forward the hypothesis that these VCFS patients could be the carriers of low activity COMT alleles on their normal chromosome 22 (Dunham et al., 1992). However, assay of COMT activity in VCFS patients who have psychiatric disorders has never been reported.

H160B/D22S66 (fig. 1A): This marker has been reported to contain a CpG island and to recognize phylogenetically conserved sequences (Driscoll et al., 1992a). Nevertheless, no data on cDNA isolation have ever been reported.

HP500/D22S134 (fig. 1B): This probe detects sequences in a range of mammals and in chick, even at high washing stringency (Wadey et al., 1993). However, several screens of mouse embryo and human fetal brain cDNA libraries did not yield any positives. Whole

sequencing of the probe revealed an homology of low significance with the human and mouse procollagen α2 (IV) chain precursor (42% identity and 68% similarity over a stretch of 38 amino acids). It was suggested that this putative HP500 coding sequence could intervene in the less frequent features of the DGS phenotype. In addition, it has been reported that collagens can promote neural crest cell migration in chicks and may function as a morphogenetic signal.

T10 (fig. 1B): T10 is a 1.5 kb cDNA isolated from a 8.5 day mouse embryonic library (Halford et al., 1993b). It maps back to a single locus in the appropriate region of 22q11.2, but no human cDNA could be found in the libraries screened. T10 codes for a potential protein of 276 amino acids, with the transcription going from telomere to centromere. No strong homologies were identified by database searching, but the predicted protein is mostly hydrophilic and serine-threonine rich. Expression studies on human tissue Northern blots reveals highest expression of a 2 kb transcript in fetal liver and low expression in fetal lung, heart and kidney. Tissue sections hybridizations of T10 on 8.5 to 15.5 days mouse embryos shows that it is expressed during early embryogenesis, in several tissues including mouth, trachea, lung, velo-pharyngeal region, arterial trunk, liver, lower limbs, etc... Although deleted in 78/80 DGS cases and 16/21 VCFS cases, T10 is not part of the DGS critical region. It is recognized that T10 is not the major gene involved in DGS, but haploinsufficiency for this gene might bring about variability in the 22q11.2 deletion phenotype.

Glycoprotein Ibβ (fig; 1A): Glycoprotein Ib (GpIb) is the major platelet receptor for von Willebrand factor. Defects in this receptor results in the autosomal recessive Bernard-Soulier syndrome (BSS), which is characterized by prolonged bleeding times, thrombocytopenia and very large platelets. GpIb is a dimer made of 2 plasma membrane glycoproteins (Ibα and Ibβ), one of which (GpIbβ) maps within the DGCR (Budarf et al., 1994b). A patient was reported with both the features of BSS and VCFS, and deletion for probe N25, thereby suggesting that haploinsufficiency for that region unmasked an autosomal recessive disorder.

TUPLE1 (fig. 1B): TUPLE1 has been isolated with probe F5 (which cross-hybridized with rodent sequences) from a human fetal brain cDNA library (Halford et al., 1993a). It recognizes a 3.4 kb transcript in all human fetal tissues tested, in addition to a 3.2 kb one in fetal liver. Sequencing predicts an open reading frame which could be 766 amino acids long, with transcription direction from telomere to centromere, giving a protein with a predicted molecular weight of 84 kD. A mouse cDNA which has 96% homology with TUPLE1 in the N-terminal part of the protein, has been isolated. Based on sequence and database searches, TUPLE1 is thought to be a transcriptional regulator: it is serine-proline rich, has a nuclear localization signal and the C-terminal end of the protein is rich in polar amino acids. More importantly, TUPLE1 shares significant similarity to the Tup1 regulatory gene of the yeast Saccharomyces cerevisae, essentially confined in the WD40 domain of the latter, and to other proteins with WD40 motifs. The WD40 repeat domain has first been found in a GTP-binding protein, the β-subunit of transducin. TUPLE1 maps 100 kb distal to the ADU balanced translocation breakpoint (P.J. Scambler, 26th meeting of the Eur. Soc. of Hum. Genet., Paris, 1994) and it has been proposed to be at least partly responsible for the phenotype of DGS and related syndromes, particularly because it is deleted in all patients known to have a deletion, and also by virtue of its potential function as a transcriptional regulator. It is postulated that the balanced translocation could exert a position effect, to explain involvement of this gene in the ADU phenotype.

N25/D22S75 (fig. 1A): N25 is a Not I linking clone which contains a CpG island and hybridizes to rodent sequences at low stringency (McDermid et al., 1989). A single cDNA was isolated from either a human fetal brain or human fetal liver library. This clone recognized a 5.5 kb transcript in adult skeletal muscle and upon sequencing, showed no obvious homology to other sequences in the databases (Emanuel et al., 1993).

Many genes from the proximal part of chromosome 22 have been mapped to mouse chromosome 16 (Bucan et al., 1993). The syntenic region extends from the immunoglobulin lambda constant region (IGLC), which maps distal to the DGS deleted region, up to

TUPLE1, and includes the T10 and COMT genes (Halford et al., 1993b; Mattei et al., 1994). No developmental mutants with a phenotype reminiscent of DGS are known to map to this region.

4. CATCH 22 as a defect involving one major gene

Many lines of evidence suggest that although most CATCH 22 patients have a very large deletion, involving loss of many genes, the genetic defect underlying this group of syndromes involves one major gene.

First, the study of other microdeletion syndromes is useful to predict the genetic defect in the CATCH 22 syndrome. For example, in the Prader-Willi/Angelman syndromes, 75% of patients have a large deletion in 15q11-q13, about 3 to 5 Mb long (Nicholls, 1993). However, some rare patients have been ascertained that have smaller deletions, and these have been useful to isolate potential candidate genes for these 2 diseases (Nicholls, 1993; Buxton et al., 1994). The same picture seems to emerge for the Miller-Dieker syndrome (MDS). Ninety percent of patients have lost a critical region estimated to 350 kb in 17p13, although two variant deletions allowed the isolation of the *LIS-1* gene, thought to be etiological in MDS (Reiner et al., 1994). Furthermore, a great majority of patients with Charcot-Marie-Tooth disease type 1A (CMT1) have a large 1.5 Mb duplication in 17p11.2-p12. On the other hand, point mutations in two CMT1 families permitted to designate the PMP22 gene as having a primary role in this pathology (Patel, 1993). Therefore, the large size of deletions observed in CATCH 22 does not preclude this syndrome to be caused by a few or even, only one gene(s).

Second, many authors have reported the lack of correlation between the severity of the phenotype, or the number of structures affected, and the size of the deletions. In addition, a number of familial cases of CATCH 22 has been reported, in which there exists a wide intrafamilial variability in the phenotype (Strong, 1968; Steele et al., 1972; Raatikka et al., 1981; Atkin et al., 1982; Miller et al., 1983; Rohn et al., 1984; Williams et al., 1985; Keppen et al., 1988;

Stevens et al., 1990; Wilson et al., 1991, 1992b, 1993a; McLean et al., 1993; Holder et al., 1993). Among others, discordance between generations for the cardiac defect or palatal anomaly were often observed. When many children from a sibship are affected, they also show very different phenotypes. In some of these families, a molecular deletion in 22q11.2 was confirmed in both the affected parent and children. However, it cannot be excluded that there is an ascertainment bias in these families, since severe phenotypes can be associated with a decreased reproductive success. But, it can be assumed that the same genetic defect was passed on from parent to children and that the variability of expression is due to the genetic background of the individual or to environmental factors.

Thirdly, the common embryologic origin of the structures affected (the 3rd and 4th pharyngeal pouches) provide another argument to suggest that the CATCH 22 syndrome can be caused by haploinsufficiency for one gene.

Finally, there has been one single report of a mother and daughter, both bearing a balanced t(2;22)(q14.1;q11.1) translocation, and displaying a DGS/VCFS phenotype (Augusseau et al., 1986). The DGS patient (ADU) has an aortic coarctation, a mild reduction in T cells, recurrent upper respiratory tract infections and otitis media, a mild neonatal hypocalcemia and a mild facial dysmorphism (microretrognathia, telecanthus, small low-set ears). The mother was considered normal at the time of publication of the report, but in retrospect, has an hypernasal speech, micrognathia and an inverted T4/T8 ratio. No information on ADU's brother, who bears the same translocation, is available. The maternal aunt, also carrier of the t(2;22) translocation, had 3 previous child and a 4th pregnancy terminated due to the presence of a cardiac defect and other anomalies in her fetus. It seems therefore possible that the translocation breakpoint interrupts the major gene involved in the pathogenesis of DGS. The few patients with no deletions in the 22q11.2 region (Scambler, 1993; Emanuel et al., 1993; and unpublished observation) could be pivotal in the identification of the CATCH 22 gene, if point mutations or small rearrangements within one gene are found.

CHAPTER 2

2.1 Introductory comment

The first part of my research project involved the search for chromosome 22q11.2 deletions in DGS patients. The aim of this study was to assess the frequency with which deletions are found in DGS, and to determine a shortest region of overlap for the different deletions in which genes potentially involved in the etiology of DGS could be isolated. The efficiencies of fluorescence *in situ* hybridization and karyotypic analysis to detect deletions were also compared. Probes were obtained form different sources: cosmids isolated with single-copy probes, Not I linking clone libraries, cosmids obtained by homology screening, or were provided by another laboratory.

2.2 Molecular cytogenetic analysis of a series of 23 DiGeorge syndrome patients by fluorescence in situ hybridization.

Abstract

We have studied a series of 23 DiGeorge syndrome patients by prometaphase chromosome analysis and/or by FISH with a set of 6 cosmid probes spanning the previously described commonly deleted region. Four patients display a cytogenetically visible interstitial deletion in band 22q11.2, whereas the other 18 patients exhibit a molecular deletion evidenced only by FISH analysis. For 21 of the patients studied, the deletion encompasses the 6 loci tested, while for one, only the most telomeric of these loci is conserved. The last patient does not show any deletion with the probes used.

Résumé

Nous avons étudié 23 patients atteints de syndrome de DiGeorge par analyse chromosomique prométaphasique et par la technique d'hybridation *in situ* fluorescente. Six sondes cosmidiques couvrant la région communément délétée ont été utilisées. Quatre patients ont une délétion de la bande 22q11.2 visible cytogénétiquement, tandis que les 18 autres patients présentent une délétion moléculaire, détectable uniquement par hybridation *in situ*. Pour 21 patients, la délétion comprend les 6 loci analysés, alors que pour un autre, le locus le plus télomérique demeure conservé. Le dernier patient n'est délété pour aucune des sondes utilisées.

Introduction

DiGeorge syndrome (DGS) is a disorder characterized by absent thymus and parathyroids, conotruncal heart defects and facial dysmorphologies (reviewed in Greenberg 1993). The complete form of DGS, as outlined below, is rare and presents a poor prognosis, but patients with milder cardiopathies, thymic hypoplasia and/or hypocalcaemia are now recognized as partial DGS and present a better prognosis. These patients account for the main part of the ascertainment in the studies recently published on DGS.

Cytogenetic analyses have revealed chromosome abnormalities in 15 to 20% of DGS cases (reviewed in Greenberg et al., 1988). The main chromosome aberrations observed were unbalanced translocations resulting in monosomy for the 22pter-> 22q11.2 region, and leading to the proposal that DGS could be a contiguous gene syndrome (Schmickel, 1986; Emmanuel, 1988). Although cytogenetic abnormalities are not detectable in a majority of DGS cases, molecular investigations of these patients with gene dosage analyses and/or RFLP studies (Scambler et al., 1991; Carey et al., 1992; Driscoll et al., 1992a; Driscoll et al., 1993) or with fluorescence *in situ* hybridization (FISH) (Desmaze et al., 1993a) have demonstrated loss of sequences in the 22q11.2 region. Similar deletions have now been described in closely related disorders such as the Shprintzen syndrome (Velo-Cardio-Facial syndrome or VCFS) (Scambler et al., 1992; Driscoll et al., 1992b; Kelly et al., 1993; Holder et al., 1993) and familial congenital heart disease (Wilson et al., 1992a; Goldmuntz et al., 1993).

We have previously shown by FISH that all affected members of a small series of four DGS families exhibit a large interstitial deletion in band 22q11.2 encompassing the loci recognized by a set of 4 cosmid probes : Sc11.1, 48F8, 100C10 and Not 54 (Desmaze et al., 1993b). We found this deletion bounded proximally by D22S9 and distally by ZNF70 as both loci are conserved in all patients studied. We now report on a series of 23 sporadic DGS cases, analyzed with the same set of probes and with two additional cosmid probes, Not

3F4 and Cos 40, that we have recently localized in the DiGeorge deleted region.

Patients, Material and Methods

Patients

Most of the patients were ascertained through their congenital heart defect. The diagnosis of DGS was retained if the patient displayed at least two of the main clinical features of DGS (congenital heart defect, hypoplastic thymus and/or parathyroids, immunodeficiency, typical facies). The main clinical and biological data are summarized in Table I. Patient ICP24 who has 0% of CD3+ lymphocytes was the only complete DGS case referred to us. Patients ICP1, ICP2 and ICP3 are patients 1, 2 and 10 from a previously published series, analyzed with the single cosmid probe Sc11.1 (Desmaze et al., 1993a). Patients ICP1 to ICP18 and ICP24 have been partially described (Aubry et al., 1993). In this study, patients ICP1 to ICP18 were all shown to be hemizygous for a zinc finger gene, ZNF74, localized distal to the recurrent t(11;22) translocation. This locus is the most telomeric described to date in the DGS region.

In situ hybridization

Chromosome preparations

Prometaphases were obtained from PHA-stimulated blood lymphocytes, after thymidine synchronization and BrdU incorporation during the last seven hours, according to usual techniques. For patient ICP24, the analysis was performed on primary fibroblast cultures.

Molecular probes

The six probes used for FISH are all cosmid clones. Sc11.1, kindly provided by P. Scambler, corresponds to the locus KI506 (Carey et al., 1990) and probes two different loci which are between 1 or 2 Mb apart in the DGS deleted region (Halford et al., 1993a; Scambler 1993). Cosmid probes 48F8, 100C10 and Not 54 have been

previously described (Desmaze et al., 1993b). All these cosmids, as well as Sc11.1, probe loci localized, with our panel of hybrid cell lines (Delattre et al., 1991), in the DGS region between the breakpoint of the 33-11-Tg cell line and that of the t(11;22) ALE cell line. Cos 40 (previously named cosDs in Desmaze et al., 1993b) has been isolated from the chromosome 22 enriched library LL22NC01 constructed in Livermore and contains the ZNF74 locus. Not 3F4 is a Not1 linking clone isolated by Sanson et al. (1993), which maps also between the 33-11-Tg and the t(11;22) ALE hybrids breakpoints. All these cosmids probe non-overlaping loci. The relative order of some of them has been previously demonstrated (Desmaze et al., 1993b). From the centromere to the telomere, the order of the loci is: proximal Sc11.1 / 48F8 / 100C10. The Not 54 locus and the distal Sc11.1 locus are both telomeric to the 100C10 locus but their relative order remains undetermined. The Cos 40 locus is distal to all these loci, and telomeric to the recurrent t(11;22) translocation breakpoint. The hybridization efficiencies for these cosmid probes (observed labelled loci / theoretical probed loci) have been previously reported (Desmaze et al., 1993b) and are 0.83, 0.68, 0.87 and 0.79 for Sc11.1, 48F8, 100C10 and Not 54 respectively. The hybridization efficiencies for the Not 3F4 and Cos 40 probes are 0.81 and 0.94 respectively. Cosmid DNAs were extracted using the alkaline lysis procedure (Sambrook et al., 1989). DNA was labelled with biotin-14-dATP using the Bionick kit (BRL, Gaithersburg) according to the supplier's recommendations. Forty to 100 ng of labelled DNA were mixed with about 100 fold sonicated human DNA in 20 µl of hybridization buffer (2XSSC, Na2HPO4 20 mM, NaH2PO4 20 mM, 50% deionized formamide, 10% dextran sulphate, 0.1% SDS, 1% Denhardt's solution).

Hybridization and Immunodetection

Slides were pretreated with RNAse A, 100mg/ml (Sigma, St Louis) for one hour at 37°C, then dehydrated in a series of ethanol baths (50%, 75%, 100%) and denatured for 2-3min at 70°C in 2XSSC (1XSSC= 0.15 M NaCl, 0.015 M NaCitrate), 70% formamide.

The hybridization mixture was denatured by boiling for 10 min, ice-cooled and spotted onto the cytogenetic spreads. The slides were incubated overnight at 42°C in a moist chamber under a plastic coverslip. They were rinsed twice in 50% formamide 2XSSC, and then in 2 baths of 2XSSC, at 42°C each. After a preincubation in PBT (PBS, 0.1% Tween 20, O.1% BSA), the biotinylated probes were revealed by a goat anti-biotin antibody, dilution 1/100 (Vector, Burlingame) and a fluorescein conjugated anti-goat antibody, dilution 1/400 (Biosys, Compiègne). The slides were counterstained with propidium iodide and mounted in an antifade solution (Johnson and Nogueira,1981).

Microscopic observations

The slides were observed on an Aristoplan Leitz microscope with a standard FITC filter combination. A mean number of 20 metaphases per patient were scored for each probe. Photographies were taken with Kodak Ektachrome 400 film.

Results

Cytogenetic analysis

Good quality R-banded prometaphases (at least 850 band stage) were obtained for all patients except ICP3, 14, 15, 20, and 23. Patient ICP24 was analyzed on fibroblast mitoses. Interstitial deletions in band 22q11.2 have been previously detected, at the cytogenetic level, for patients ICP1 and ICP2 (Desmaze et al., 1993). Deletions in the same band were observed for patients ICP4 and ICP11 (Fig. 1). The remaining patients have neither cytogenetic microdeletions nor any detectable chromosome aberrations.

FISH

Deletion of a given locus is demonstrated by the labelling of a single chromosome 22 (Fig. 2). The results obtained by FISH are summarized in table 2. The first twelve patients ascertained were studied with all six probes. As data on the physical order of the probes began to accumulate (Lindsay et al., 1993; Desmaze et al., 1993b), and with the assumption that, whenever two loci are lost, the DNA segment in between is also deleted, the remaining patients were studied with a subset of the cosmid probes. Thus, seven cases were analyzed with five probes, one patient with four probes, two with three probes and one with two probes. A deletion was observed in 22 of the 23 patients. In all patients but two (patients ICP24 and ICP37), all the loci probed were found deleted on one of the chromosome 22. Patient ICP37 was found deleted for the six more proximal loci tested. Therefore, these results strongly suggest that all patients but one (ICP24) carry a large deletion in the 22q11.2 region, as previously observed in the familial cases we studied before (Desmaze et al., 1993b).

Discussion

In this series of 23 sporadic DGS patients, a microdeletion is detected at the cytogenetic level in 4 of the 17 patients studied with a high resolution banding technique. This frequency (.24) is comparable to those already estimated (Greenberg et al., 1988; Driscoll et al., 1992a; Wilson et al., 1992b). Apart from the exclusion of translocations or chromosome rearrangements, prometaphase analysis is of limited usefulness in the diagnosis of DGS, as it only detects a small proportion of chromosome 22 deletions. FISH analysis appears as a more convenient and powerful tool to detect these microdeletions and to determine their extent, as previously proposed by us and others.

In our series, an interstitial deletion in band 22q11.2 is demonstrated by FISH in all cases but one. In the 12 patients studied with our complete set of cosmids, the deletion encompasses the seven loci probed. These patients are thus deleted for a large DNA region that extends from proximal Sc11.1 to ZNF74 (the most proximal and distal loci mapped in the DGS region, respectively). The other 10 patients, although less extensively studied, are also deleted for all the loci probed.

Among the 22 patients with demonstrated deletions, 21 have been studied with the cosmid probe Sc11.1. All these 21 patients are hemizygous for the two loci recognized by this cosmid, which are about 1-2 Mb apart. If we add to this series 12 previously published cases (Desmaze et al., 1993a; Desmaze et al., 1993b), 33 of our 34 patients analyzed with Sc11.1 are deleted for these two loci. Among these patients, 19 have been analyzed with the Cos 40 probe and all but one (ICP37) are hemizygous at this locus (ZNF74). It is noticeable that patient ICP37 is the only one from our series with no cardiopathy. This patient has a normal development, no dysmorphic features and presents a mild immunodeficiency and a hypoparathyroidy. However, it is unlikely that this patient's mild phenotype can be explained by the lesser telomeric extent of the deletion, since DGS patients with severe cardiopathy and deletion

borders mapping much more proximally have been reported before (Carey et al., 1992; Driscoll et al., 1992).

With the exception of ICP37, no difference for the extent of the deletions has been evidenced among our patients. This finding differs from that obtained by others who have observed, in their series, frequent variations of the proximal boundaries (Driscoll et al., 1992a) and of the distal boundaries (Carey et al., 1992; Driscoll et al., 1992a) of the deletions. These discrepancies could eventually be due to differences between the technical approaches used to demonstrate loss of loci. It is noticeable that our study is the first one performed by FISH only and that, with the exception of Sc11.1 which recognizes two loci, all the cosmids used in our analysis probe single loci. Therefore, in our series, it is not possible to correlate the severity of the disorder with either the extent of the deletion at the molecular level or the existence of a cytogenetically detectable deletion. This observation is against the suggestion that DGS could be a contiguous gene syndrome. Conversely, it is surprising that in our series the only patient with typical complete DGS (ICP24) does not exhibit a loss of any of the six loci tested. The disorder observed in this patient could be the result of another developmental defect than the large 22q11.2 deletion usually observed.

All the patients studied in our series have been referred to us as DGS. None present a patent cleft palate or additional malformations evoking a VCFS. However, the typical facial features of this syndrome are indeed difficult to recognize in newborn children, in particular when the initial clinical examination is performed in an intensive care unit. In the literature, some patients initially presented as DGS have been secondarily classified as VCFS, and we cannot exclude this possibility for some patients in our series. With the probes available to date, molecular analysis do not allow to distinguish between the two syndromes since very similar deletions are observed.

From our results and from previously published data (Scambler et al., 1991; Carey et al. 1992; Driscoll et al., 1992a; Desmaze et al., 1993a; Scambler, 1993; Desmaze et al., 1993b), it appears that almost all patients ascertained for DGS present a large deletion in band

22q11.2. Accumulating data on the physical map of the DGS region demonstrates that such a large deletion is not necessary to express the cardinal features of DGS and that the region which when lost gives the syndrome (smallest deleted region) maps to the centromeric part of the large deletion (Lindsay et al., 1993; Desmaze et al., 1993b). The fact that such a large deletion is observed in almost all patients studied could be explained by the presence within the 22q11.2 region of low copy number repeat sequences that could favour deletions through recombination events, as previously proposed by Halford et al. (1993a).

We demonstrate here that, as for the familial DGS cases (Desmaze et al., 1993b), the gene encoding the COMT (included in cosmid 100C10) is deleted in all the 21 sporadic DGS patients studied but one. As previously proposed (Dunham et al., 1992; Scambler 1993), deletion of this locus could be relevant for some neurological aspects of DGS related disorders, VCFS in particular. There exists two alleles of this enzyme (low and high activities). If we assume that about 25% of the general population has a low COMT activity and that almost all DGS patients are deleted at this locus, a large proportion of DGS patients would have an extremely reduced COMT activity and thus should present, at an older age, the same psychotic features than those reported in VCFS. However, behavioural disorders have never been reported in association with DGS. Due to progress in cardiac surgery and to the inclusion in the DGS designation of milder forms, more patients should reach adult age and thus be more susceptible to present psychotic disorders.

Three sequences potentially located in the 5' region of genes [the NotI linking clone N25 (Driscoll et al. 1992), the NotI linking clone Not 54 (Desmaze et al., 1993b) and the locus pH160b containing a CpG island (Driscoll et al. 1992)], a fetal brain cDNA (Lamour et al., 1993), a zinc finger gene, ZNF74 (Aubry et al., 1993), a gene expressed in early embryogenesis (Halford et al., 1993b) and a gene encoding a putative transcriptional regulator (Halford et al., 1993c) have been previously assigned to the DGS commonly deleted region in band 22q11.2. However, only the N25 locus and the gene encoding the putative transcriptional factor have been mapped in the smallest

deleted region. We localize here another NotI linking clone, Not 3F4, in the DGS commonly deleted region. The precise sublocalization and the demonstration that this locus really contains coding sequences is under study. It would be of great interest to determine the eventual involvement of these various loci in the occurrence of the major or minor signs of DGS.

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Figure 1

Prometaphase chromosomes 22 from ICP4 (case 1) and ICP11 (case 2) patients with normal chromosomes 22 on the left and deleted chromosomes 22 on the right. Arrows point to the deleted regions.

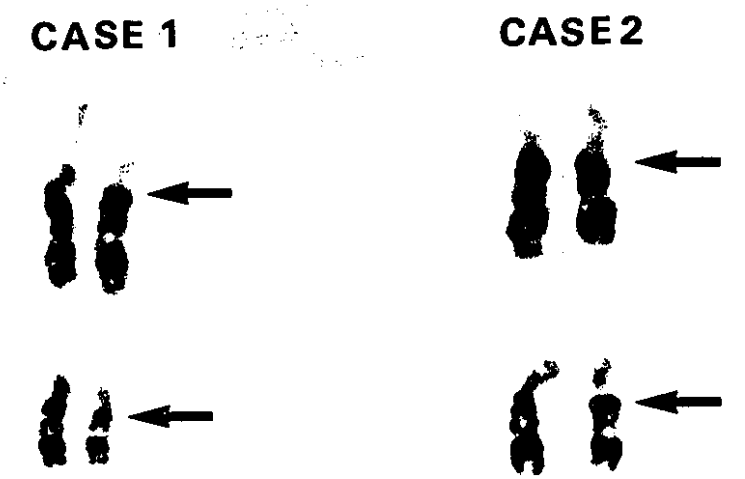

Figure 2

Fluorescence in situ hybridization of cosmid 48F8 on metaphase from ICP37. Arrow points to the normal chromosome 22.

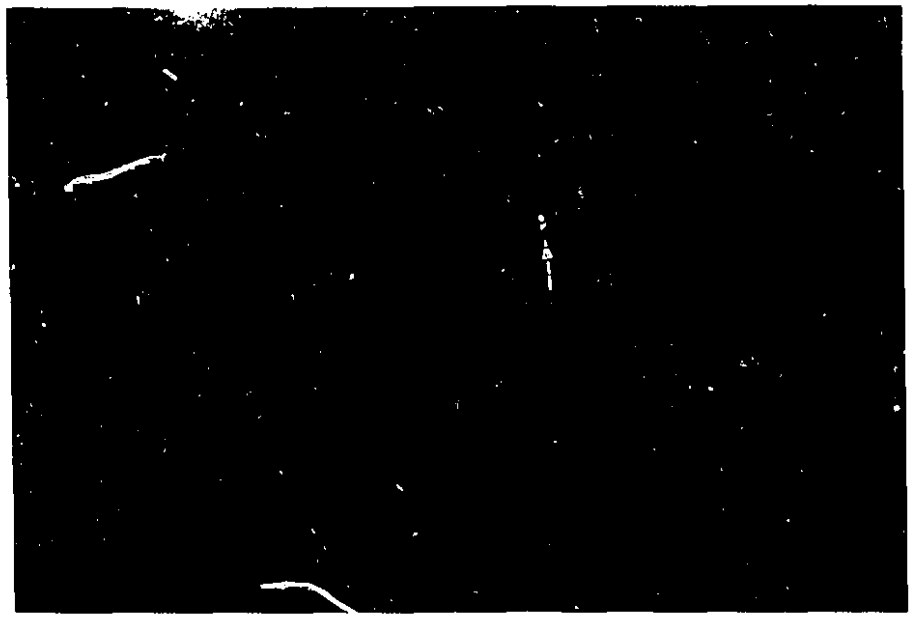

Table 1

Main clinical and biological observations on the 23 DGS patients.

Abbreviations used:

VSD: ventricular septal defect, AAA: aortic arch anomaly, IAA: interrupted aortic arch, TA: truncus arteriosus, PA: pulmonary atresia, IAC: interauricular communication, Fallot: tetralogy of Fallot, ND: not determined, +: presence of the feature, -: absence of the feature.

Patients	Sex	Age (years)	Karyotype	Congenital heart-defect	Thymus	% CD3+	% CD4+	% CD8+	Hypocalcaemia	Facial dysmorphies
ICP 1	F	1	del(22)(q11.2)	IAA, VSD	ND	40	15	35	+	ND
ICP 2	M	.7	del(22)(q11.2)	Fallot	-	35	20	20	+	+
ICP 3	M	1	normal	Fallot	+	74	44	40	+	+
ICP 4	F	4	del(22)(q11.2)	Fallot	-	27	17	14	+	+
ICP 5	M	8	normal	VSD, PA	ND	58	27	16	?	+
ICP 6	M	1	normal	VSD, PA	ND	42	30	6	-	+
ICP 7	F	.4	normal	IAA, VSD	-	37	ND	ND	-	+
ICP 8	F	15	normal	Fallot	-	92	52	32	+	-
ICP 9	F	.5	normal	IAA, VSD	+	35	ND	ND	+	+
ICP 10	M	.6	normal	VSD, TA	-	ND	ND	ND	+	+
ICP 11	M	2	del(22)(q11.2)	VSD, PA	-	95	53	37	-	+
ICP 12	M	5	normal	IAA, VSD	-	55	37	25	+	+
ICP 13	F	.5	normal	TA	+	ND	ND	ND	-	+
ICP 14	M	1	normal	VSD	-	ND	ND	ND	-	+
ICP 15	M	1	normal	VSD	+	ND	ND	ND	-	+
ICP 16	M	1	normal	IAA, VSD	-	45	34	28	-	-
ICP 17	M	4	normal	AAA, TA	-	51	25	16	-	+
ICP 18	F	.9	normal	VSD, AAA	-	ND	ND	ND	ND	+
ICP 24	M	.1	normal	IAC, PA	-	0	ND	ND	+	+
ICP 30	F	8	normal	Fallot	ND	60	33	21	-	+
ICP 31	F	.1	normal	Fallot	-	52	41	15	-	+
ICP 32	F	.1	normal	IAA, VSD	-	50	30	42	-	+
ICP 37	M	.2	normal	None	-	25	16	33	+	-

Table 2

FISH results obtained with the 6 probes.

Abbreviations used:

Del: deleted locus, Not Del: conserved locus, ND: not determined.
The previously published data pertaining to Cos 40 for patients ICP1 to ICP18 and ICP24 are reported here for a more comprehensive overview.

PATIENTS	SC11.1	48F8	100C10	NOT54	NOT3F4	Cos40
ICP1	Del	Del	Del	Del	Del	Del
ICP2	Del	Del	Del	ND	Del	Del
ICP3	Del	Del	Del	Del	ND	Del
ICP4	Del	Del	Del	Del	Del	Del
ICP5	Del	Del	Del	ND	Del	Del
ICP6	Del	Del	Del	Del	Del	Del
ICP7	Del	Del	Del	Del	Del	Del
ICP8	Del	Del	Del	Del	Del	Del
ICP9	Del	Del	Del	Del	Del	Del
ICP10	Del	Del	Del	Del	Del	Del
ICP11	Del	Del	Del	Del	Del	Del
ICP12	Del	Del	Del	Del	Del	Del
ICP13	Del	Del	Del	Del	Del	Del
ICP14	Del	Del	Del	Del	Del	Del
ICP15	Del	Del	Del	ND	ND	Del
ICP16	Del	Del	Del	Del	Del	Del
ICP17	Del	Del	Del	ND	Del	Del
ICP18	Del	Del	Del	ND	Del	Del
ICP24	Not Del	Not Del	Not Del	ND	Not Del	Not Del
ICP30	Del	Del	Del	ND	ND	ND
ICP31	Del	Del	Del	ND	ND	ND
ICP32	ND	Del	Del	ND	ND	ND
ICP37	Del	Del	ND	Del	Del	Not Del

CHAPTER 3

3.1 Introductory comment

A correlation between the size of the DGS deletion and the phenotype does not appear to emerge in the patient series we and others studied. In contrast, the concept that the parental origin of the deletion could modulate the variable expressivity of the phenotype is very attractive. An excess of transmitting mothers in familial and sporadic CATCH 22 has been reported in a number of studies, but the sample sizes were small. In our own familial cases, the deletion was inherited form the mother in 4 out of 5 cases. Because we had isolated a CA-repeat loci mapping within the DGS deleted region, the parental origin of the deletion was determined in 29 sporadic DGS pedigrees by assessing the inheritance pattern of this microsatellite locus, and by using a chromosome 22-short arm heteromorphism.

3.2 Excess of deletions of maternal origin in the DiGeorge/Velo-Cardio-Facial syndromes. A study of 22 new patients and review of the literature.

Abstract

We have determined the parental origin of the deleted chromosome 22 in 29 cases of DiGeorge syndrome (DGS) using a CA-repeat mapping within the commonly deleted region, and in one other case by using a chromosome 22 short arm heteromorphism. The CA-repeat was informative in 21 out of 29 families studied and the deleted chromosome was of maternal origin in 16 cases (72%). When these data are pooled with recent results from the literature, 24 de novo DGS, Velo-Cardio-Facial syndrome (VCFS) and isolated conotruncal cardiac disease deletions are found to be of maternal origin and 8 of paternal origin, yielding a X^2 of 8 with a probability level lower than 0.01. These data, and review of the literature on familial DGS/VCFS and isolated conotruncal cardiopathies suggest that there is a strong tendency for the 22q11.2 deletions to be of maternal origin.

Introduction

DiGeorge syndrome (DGS) is a developmental defect of the third and fourth pharyngeal pouches which associates hypo- or aplasia of the thymus and parathyroids, and conotruncal cardiac malformations. The etiological factor in a great majority of DGS cases is monosomy for the 22q11.2 chromosomal region either through a large interstitial deletion of that region (inherited or de novo) or through an unbalanced translocation involving chromosome 22 (either inherited or not) (de la Chapelle et al., 1981; Kelley et al., 1982; Bowen et al., 1986; Faed et al., 1987; Schwanitz and Zerres, 1987; Dallapiccola et al., 1989; Greenberg et al., 1988; Mascarello et al., 1989; Pinto et al., 1989; Pivnick et al., 1990; El-Fouly et al., 1991; Lupski et al., 1991; Carey et al., 1992; Driscoll et al., 1992a; Desmaze et al., 1993a).

Deletions involving the same chromosomal region as DGS have been recently reported for other closely related syndromes such as the velo-cardio-facial syndrome (VCFS) (Driscoll et al., 1992b; Kelly et al., 1993; Scambler et al., 1992), isolated conotruncal cadiac defects (either sporadic or familial) (Wilson et al.,1991; Goldmuntz et al., 1993), the CHARGE association (Clementi et al, 1991; Emanuel et al., 1992) and the conotruncal anomaly face syndrome (Burn et al., 1993), which prompted one group to coin the acronym CATCH 22 (Cardiac defect, Abnormal facies, Thymic hypoplasia, Cleft palate, Hypocalcaemia, chromosome 22q11 deletions) to designate this group of related syndromes (Wilson et al., 1993).

The concept that the parental origin of the deletion in sporadic DGS or VCFS can modulate the variable expressivity of the phenotype has been proposed before and is attractive (Hall, 1990, 1993; McLean et al., 1993; Driscoll et al., 1993; Holder et al., 1993). The parental origin of the deletion has been determined in 10 cases of DGS or VCFS, too small a sample to attain statistical significance (Greenberg et al., 1988, Driscoll et al., 1992a, b; Seaver et al., 1994).

We have determined the parental origin of the deleted chromosome 22 in 22 cases of DGS by assessing the parental origin of a CA-repeat locus mapping within the DGS deleted region, and by

using a cytogenetic heteromorphism on the short arm of chromosome 22. We present evidence that the chromosome bearing the deletion in sporadic DGS cases is predominantly of maternal origin.

Material and Methods

The DGS cases were ascertained on the basis of the presence of a conotruncal cardiopathy, and at least one of the following features: thymus and/or parathyroids hypoplasia and facial dysmorphologies. DNA from the patients and their parents was extracted by standard methods. Most of the patients have been studied before either by FISH or Southern dosage studies, and bear proven deletions in the 22q11 region. The primers for the CA-repeat cos 39 have been published before (Marineau et al., 1992) with accession number EMBL X62391.

This CA-repeat sequence has been identified in a cosmid containing a zinc finger gene which has been localized on chromosome 22 between the t(11;22) constitutional recurrent breakpoint and the t(9;22) chronic myeloid leukemia breakpoint (Aubry et al., 1993). This cosmid locus was found deleted in 27 out of 30 DGS cases studied with normal chromosome complements.

PCR was performed in a volume of 25 µl containing 125 ηg of human genomic DNA, 15 pmoles of each primer, 1.5 mM $MgCl_2$, 50 mM KCL, 10 mM Tris-HCL, pH8.3, 200 µM of each of the 4 dNTP and 0.625 unit of Taq DNA polymerase in a 9600 Perkin-Elmer Cetus PCR apparatus. PCR conditions were as follow: initial denaturation (94°C-5 minutes) followed by 35 amplification cycles (94°C-30 seconds, 55°C-30 seconds, 72°C-1 minute) and a final elongation (72°C-5 minutes).

The denatured PCR products (approximately 200 bp) were electrophoresed on a 6% polyacrylamide denaturing gel, transferred onto Hybond N^+ nylon membrane for at least 2 hours and hybridized at 42°C (minimum 2 hours) with a $(CA)_{12}$ radiolabelled probe using a terminal transferase kit according to manufacturer's protocol (Boehringer-Mannheim), in hybridization buffer (0.13 M Na_2HPO_4/NaH_2PO_4, 10% PEG 6000, 7% SDS, 0.25 M NaCl). Membranes were washed in 2XSSC at room temperature and exposed with autoradiographic films for a few hours. Alleles were scored by attributing them a number depending on size, within a given family. The genotypes were derived from two independent readings of the autoradiograms.

Results

Table 1 summarizes the results of the D22S264 CA-repeat analysis and clinical features of the patients. Out of 29 families studied, the CA-repeat cos39 was informative in 21 cases (72%). Figure 1 shows a representative CA-repeat analysis on DNA from a DGS patient and parents. In 14 families, both parents were heterozygous and did not share an identical allele. In only 4 instances, one parent exhibited a single allele, an observation compatible with homo- or hemizygosity at this locus. However, in 3 cases, the absence of a deletion for probes in the DGS region was confirmed by fluorescence in situ hybridization (FISH) on chromosome preparations from the parent. In one family (MAR 7), DNA from the father was not available. Nevertheless, the mother was heterozygous and found by FISH not to be deleted for the loci probed by Sc4.1 and Sc11.1 (Carey et al., 1990, 1992). The DGS child did not inherit a maternal allele and was deleted for probes Sc4.1 and Sc11.1 by FISH. We can thus conclude that in this case the deletion is of maternal origin.

In one case, the parental origin of the deleted chromosome was determined using a cytogenetic length heteromorphism of the short arm of chromosome 22 (case 1 of Desmaze et al., 1993a). The patient has a cytogenetically visible interstitial deletion on the chromosome 22 that displays the shortest p-arm. The mother of the patient also bears this heteromorphism on one of her chromosome 22 short arm. Therefore in this case the deletion is of maternal origin.

Altogether, we found the deletion to be of maternal origin in 16 cases and of paternal origin in 6 cases. This distribution is significantly different from a random one ($X^2 = 4.5$; $p < 0.05$).

Discussion

We show in our series of sporadic DGS that the 22q11.2 deletions are non-random, being of maternal origin in 16 out of 22 cases ($X^2=4.5$, $p < 0.05$) (table 1). The parental origin of the deleted chromosome has been previously determined in 10 other cases of sporadic CATCH 22 using RFLPs and microsatellites inheritance in the DGS commonly deleted region, and cytogenetic heteromorphisms (Greenberg et al., 1988; Driscoll et al., 1992a,b; Seaver et al., 1994). Therefore, among the 32 sporadic CATCH 22 cases examined, 24 deletions are of maternal origin and 8 of paternal origin ($X^2=8$, $p < 0.01$).

The observation of an excess of transmitting mothers in familial DGS, VCFS or congenital heart disease cases has been made previously (Shprintzen et al., 1981; Wilson et al., 1991, 1992, 1993; Driscoll et al., 1992b, 1993; Desmaze et al., 1993b). The parental origin of the deleted chromosome 22 has been determined by molecular analysis in 33 CATCH 22 families, and suspected, by the presence of some clinical features in one of the parents, in 18 others (Strong, 1968; Steele et al., 1972; Young et al., 1980; Shprintzen et al., 1981; Lagrue and Griscelli, 1984; Rohn et al., 1984; Williams et al., 1985; Wraith et al., 1985; Meinecke et al., 1986; Keppen et al., 1988; Stevens et al., 1990; Lipson et al., 1991; Scambler et al., 1991; Wilson et al., 1991, 1992, 1993; Driscoll et al., 1992b, 1993; Desmaze et al., 1993b; Holder et al., 1993; Kelly et al., 1993; McLean et al., 1993; D'Angelo et al., 1994; Demczuk et al., 1994; Hajianpour et al., 1994; Puder et al., 1994; Piussan et al., 1994; Ravnan et al., 1994). A significant excess of transmitting mothers (42 out of 51 cases) was demonstrated ($X^2=21.3$, $p < 0.0001$). Therefore, when the data of the familial cases are pooled with those observed in the de novo deletion cases we and others studied, 66 DGS/VCFS cases have only paternal alleles remaining, while 17 have only maternal alleles in that genomic region ($X^2=29$, $p < 0.0001$).

Finally, the parental origin of monosomy 22q11 has been determined in some instances of DGS arising through unbalanced translocations (De la Chapelle et al., 1981; Kelley et al., 1982;

Greenberg et al., 1984; Bowen et al., 1986; Dallapicolla et al., 1989; Pivnick et al., 1990; El-Fouly et al., 1991). Although the sample size is rather small, no departure from randomness is observed: the translocations are maternally-derived in 4 instances and paternally-derived in 4 others. Pooling these 8 DGS translocation cases with the previous data gives 70 occurrences of DGS/VCFS cases bearing only paternal alleles and 21 with only maternal alleles in the 22q11.2 region ($X^2=26.4$, $p < 0.0001$).

Therefore, when our cases and cases from the literature are examined, there appears to be a net tendency for the deletions to be of maternal origin in the CATCH 22 syndrome. Different hypotheses may account for this non random parental origin of chromosome 22 deletions. Although 91 cases/families in total have been considered, the sample size in each category remains relatively small. It would be appropriate to extend this analysis to more cases, in particular for sporadic CATCH 22. In familial cases, this preferential parental origin could be due to decreased reproductive success or decreased fertility in males. However, this hypothesis does not explain the preferential occurrence of maternal deletions in sporadic cases. Even though no imprinted genes to date have been mapped to chromosome 22 or to the synthenic region on mouse chromosome 16 (Bucan et al., 1993), another explanation could be that this chromosomal region is subjected to imprinting, i.e. a differential expression of the genes depending on their parental origin. If this hypothesis is true, the phenotypic differences between CATCH 22 of maternal and of paternal origins do not clearly emerge from the clinical data reported in the literature.

Nevertheless, about 30% of DGS cases exhibit a deletion of paternal origin. If an imprinting mechanism is involved in the CATCH 22 syndrome, then the resulting phenotype when the deletion is inherited from the father may be more severe and not compatible with survival. On the other hand, the phenotype may be so mild that children without paternal alleles are simply not ascertained. Arguments in favor of one or the other hypothesis have never been documented in CATCH 22 pedigrees and need closer examination. Assuming an imprinting mechanism, the occurrence of deletions of

paternal origin could alternatively be explained by the existence of genetic variants at loci responsible for imprinting, as previously described for other loci (IGF2 for the Beckwith-Wiedemann syndrome: Weksberg et al., 1993; loci in the Prader-Willi and Angelman syndromes deleted region: Reis et al., 1994; mouse t-locus: Forejt and Gregorova, 1992). It would, therefore, be interesting to determine whether there is a differential expression of the parental alleles for the genes located in the DGS critical region.

Finally, the higher frequency of deletions of maternal origin could be explained by some particular structural features of the chromatin allowing the maternal chromosome to be more liable to delete (e.g. differential compaction, excess of maternal non homologous recombination) or to an unexplained preferential rearrangement of the maternal chromosome 22, as observed in chronic myeloid leukaemia where the maternal BCR gene always seems to be involved in the specific translocation with ABL (Haas et al., 1992), without evidence of any functional imprinting of the two parental BCR alleles (Riggins et al., 1994).

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Figure 1

Representative example of D22S264 CA-repeat analysis in a DGS pedigree. Number on the left indicate the different alleles present in the family. The patient has inherited only allele number 2 from his father.

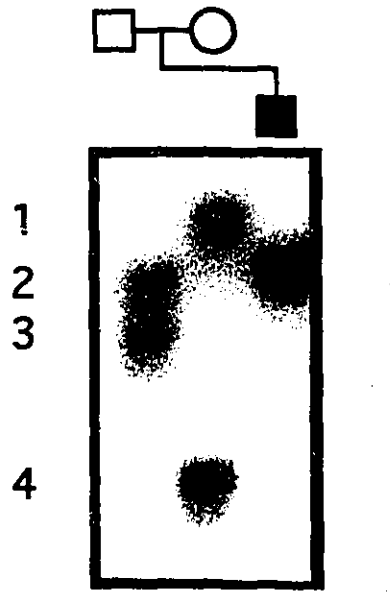

Table 1

Results of D22S264 CA-repeat analysis in the DGS families, and main clinical and biological data for the probands.

Abbrevations used:

mat: maternal; pat: paternal; ND : Not Determined. IAA: Interrupted Aortic Arch; PA: Pulmonary Atresia; VSD : Ventricular Septal Defect; RSA: Anomaly of Right Subclavian Artery; TOF : Tetralogy of Fallot; AAA : Aortic Arch Anomaly; TA : Truncus Arteriosus; ASD : Atrial Septal Defect; +: presence of the feature; -: absence of the feature; ?: unknown.

Patient number	Sex	ALLELES			Origin of deleted chr. 22	CLINICAL FEATURES				
		Patient	Father	Mother		Cardiopathy	Thymus	T-cell level (%) CD3/CD4/CD8	Hypocalcaemia	Facial dysmorphies
ICP 5	M	2	3,4	1,2	pat	VSD, PA	ND	58/27/16	?	+
ICP 6	M	2	1,2	1,3	mat	VSD, PA	ND	42/30/06	-	+
ICP 8	F	4	1,2	3,4	pat	TOF	-	92/52/32	-	+
ICP 17	M	2	2,4	1,3	mat	AAA, TA	-	51/25/16	-	+
ICP 18	F	2	1,2	3,3	mat	VSD, AAA	-	ND	ND	+
ICP 31	F	4	2,4	1,3	mat	TOF	-	52/41/15	-	+
ICP 36	M	3	2,4	1,3	pat	RSA	ND	46/?/?	+	+
ICP 42	M	1	2,4	1,3	pat	VSD	ND	74/37/26	+	+
ICP 47	M	2	1,2	1,3	mat	+	ND	72/33/30	+	?
ICP 52	M	2	2,3	1,4	mat	+	ND	71/25/34	-	?
ICP 54	M	4	2,4	1,3	mat	VSD	ND	ND	ND	+
LTM 1	F	2	1,4	2,3	pat	TOF	-	?/21/38	+	+
LTM 2	F	2	1,2	3,3	mat	TA	-	46/34/14	+	+
LTM 3	F	1	1,3	2,2	mat	TOF	-	35/23/13	+	+
LTM 4	F	3	1,3	2,4	mat	VSD, PA	+	ND	-	+
LTM 5	F	3	2,3	1,1	mat	TOF	ND	ND	ND	+
LTM 6	F	1	1,4	2,3	mat	IAA, VSD, ASD	-	59/45/13	+	+
LTM 7	F	3	ND	1,2	mat	TOF	-	38/24/14	-	+
KDH 1	M	3	1,3	2,4	mat	IAA, VSD, ASD	-	decreased	+	+
KDH 2	F	2	2,3	1,4	mat	IAA, VSD, ASD	ND	normal	ND	+
NEM 10	M	2	1,3	2,3	pat	IAA, VSD	-	ND	+	?

3.3 Appendix 1
Clinical features of the CATCH 22 patients with known parental origin of the deletion

This table presents the clinical features of the CATCH 22 patients from pedigrees reported in the literature. The information presented are as was outlined in the corresponding publication, and therefore are sometimes rather partial. Especially, it turns out to be more and more difficult to categorize a patient into a particular diagnostic label, since now patients are said to be affected with CATCH 22 or DGS/VCFS.

As can be seen from these data, no clear correlation emerges between phenotype and parental origin of the deletion. However, there appears to be a greater tendency for VCFS to be familial and to be inherited from the mother. This observation could be explained by the fact that the VCFS probably lies at the less severe end of the spectrum of malformations defined by CATCH 22, and therefore is more likely to be seen within families.

Parental origin of the deletion, and main clinical and biological data in DGS/VCFS and isolated cardiopathies cases.

Abbreviations used:
mat: maternal; pat: paternal; DGs: DiGeorge syndrome; VCFS: Velo-Cardio-Facial syndrome; CHD: Congenital heart defect; ND: not determined; NP: not patent; IAA: interrupted aortic arch; PA: pulmonary atresia; VSD: ventricular septal defect; LSA and RSA: anomaly of left or right subclavian artery; TOF: Tetralogy of Fallot; AAA: aortic arch anomaly; TA: truncus arteriosus; RAA; right aortic arch; PDA: patent ductus arteriosus; ASD: atrial septal defect; TGA: transposition of the great arteries; +: presence of the feature; -: absence of the feature; Sm. cleft palate: submucous cleft palate.

a) De novo deletions

Author Patient no. (sex)	Parental origin	Diagnosis	Thymus	T-cell level	Heart	Hypocalcemia	Mental retard.	Facial dysmorphy	Other features
Greenberg et al. (1988)									
Patient 27 (M)	mat	DGS	Absent		TA, RAA VSD	+		+	
Driscoll et al. (1992a)									
DGS 3 (M)	mat	DGS	Hypoplastic	Decreased	PDA	+		+	
DGS 4 (M)	mat	DGS	NP	ND	TOF	+			
DGS 5 (F)	mat	DGS	Hypoplastic		IAA, VSD	+		-	
DGS 9 (F)	pat	DGS	Hypoplastic		IAA, VSD	+		+	Multicystic kidney
Driscoll et al. (1992b)									
VCF-8 (F)	pat	VCFS	ND		RAA		+	+	Cleft palate
Seaver et al. (1994)									
Case 2 (F)	mat	CHD	ND		PA/VSD		ND	+	Umbelical hernia, slender fingers, hyperextensibility
Case 4 (F)	mat	CHD	ND		PA/VSD		ND	+	Hyperextensibility
Case 5 (F)	mat	CHD	ND		PA/VSD		ND	+	Hyperextensibility
Case 6 (M)	mat	CHD	ND		PA/VSD		ND	+	Umbelical hernia

b) Familial deletions (confirmed with molecular diagnosis)

Author Patient no. (sex)	Diagnosis	Thymus	T-cell level	Cardiac malformation	Hypocal-cemia	Mental retardation	Facial dysmorphy	Other features
Scambler et al. (1991) / Rohn et al. (1984)								
Father		ND	Decreased	None	+		+	
Child 1 (M)	DGS	Absent	Decreased	TA, PA	+		+	
Child 2 GM7939 (M)	DGS	Absent	Decreased	TA, VSD, PA	+		+	Cleft palate, supernumerary digits, micropenis
Scambler et al. (1991) / Keppen et al. (1988)								
Father		ND	Decreased	RAA, PDA	-		+	Cleft palate
Child (F)	DGS	Absent	Normal	IAA, PDA, RSA	+			
Wilson et al. (1991)								
Mother		ND		None			+	
Case 1 (F)	DGS	ND	Decreased	IAA, VSD	+		+	Talipes equinovarus, no left kidney
Case 2 (M)		ND	Normal	VSD	-		+	High arch palate
Case 3 (M)		ND	Normal	IAA	+		+	Talipes equinovarus
Wilson et al. 1992								
Family 1	CHD							
Mother				TOF				
Child (M)				PA, VSD				
Family 2	CHD							
Mother				RAA, PDA				
Child 1 (F)				TA	+			
Child 2 (M)				PA, VSD				
Family 3	CHD							
Mother				Cardiopathy				
Child (F)				PA, VSD				
Family 4	CHD							
Father				RAA, VSD, LSA				
Child 1 (F)				VSD, Dextrocardia				
Child 2 (M)				TOF				
Child 3 (F)				TOF				

b) Familial deletions (confirmed with molecular diagnosis) (cont.)

Author Patient no. (sex)	Diagnosis	Thymus	T-cell level	Cardiac malformations	Hypocal-cemia	Mental retardation	Facial dysmorphy	Other features
Family 5	CHD							
Father				None				
Child 1 (M)				PDA	+			Developmental delay
Child 2 (F)		Absent		IAA, VSD, RSA				
Child 3 (M)				TOF				
Driscoll et al. (1992b)								
VCF-5, **mother**	VCFS			VSD			+	Cleft palate, learning disability
VCF-4 (F)	VCFS			PDA			+	Cleft palate, learning disability
VCF-10, **mother**	VCFS			None			+	Cleft palate, learning disability
VCF-11 (F)	VCFS			None			+	Cleft palate
Desmaze et al. (1993b)								
A II1 **mother**		ND	ND	None	+	+	+	
A III2 (F)	DGS	Absent	Normal	None	+	+	+	Neurologic disorders, swallowing difficulties
B II1 **mother**		ND	ND	None	ND	+	+	
B III1 (F)	DGS	Absent	Decreased	IAA	+	+	+	Swallowing difficulties, tapered fingers and toes
B III2 (M)		ND	Decreased	IAA	ND			Not born
C II2 **father**		ND	Normal	None	+	-	+	
C III2 (M)	DGS	Present	Normal	AAA	-	+	+	Hypernasal speech,
C III3 (M)	DGS	Present	Normal	PA, VSD	-	+	+	Hypernasal speech, partial hearing loss
D II1 **mother**	VCFS	ND	ND	None	ND	+	+	Cleft uvula
D III1 (M)	DGS	Hypoplastic	ND	PA, VSD	ND		+	
D III2 (F)	DGS	Absent	ND	TA, VSD	ND		+	

b) Familial deletions (confirmed with molecular diagnosis) (cont.)

Author Patient no. (sex)	Diagnosis	Thymus	T-cell level	Cardiac malformation	Hypocal-cemia	Mental retardation	Facial dysmorphy	Other features
Driscoll et al. (1993)								
Family A								
Father								Learning disability
Child (M)	DGS	Hypoplastic	ND	Cardiopathy	+			
Family B								
Father								Learning disability
Child 1 (M)	DGS	Hypoplastic	ND	Cardiopathy	+			
Child 2 (M)	DGS	Hypoplastic	ND	Cardiopathy	+			
Family C								
Mother								Learning disability
Child (M)	DGS	Hypoplastic	ND	Cardiopathy	+			
Family D								
Mother								Cleft palate, learning disability
Child (M)	DGS	Hypoplastic	ND	Cardiopathy	+			
Family E								
Mother	VCFS			Cardiopathy			+	Cleft palate, learning disability
Child (M)	VCFS			Cardiopathy			+	Cleft palate, learning disability
Holder et al. (1993)								
Mother	VCFS			Cardiopathy				Cleft palate, hypernasal speech
Child (F)	VCFS			None				
Kelly et al. (1993)								
Mother, NW 13	VCFS			None				Cleft palate
Child, NW 14 (F)	VCFS			VSD, ASD				

b) Familial deletions (confirmed with molecular diagnosis) (cont.)

Author Patient no. (sex)	Diagnosis	Thymus	T-cell level	Cardiac malformation	Hypocal-cemia	Mental retardation	Facial dysmorphy	Other features
Mc Lean et al. (1993)								
Mother	VCFS			None			+	Cleft palate, learning disability, tapered fingers
Child (M)	VCFS			TOF			+	Learning disability, tapered fingers
Wilson et al. (1993)								
Family 1 **mother deleted**	DGS							
Family 2 **mother deleted**	DGS							
Family 3 **mother deleted**	DGS							
Family 4 **mother deleted**	DGS							
Demczuk et al. (1994)								
Mother				Cardiopathy				
Child ICP11 (M)	DGS	Absent	Normal	PA, VSD	-		+	
Piussan et al. (1994)								
Mother	VCFS (typical)						+	
Child 1 (F)					+	+	+	Noonan-like, hemivetebrae
Child 2 (F)				PVS	+	+	+	renal malformation
D'Angelo et al., (1994)								
Mother	VCFS			PS/RAA			+	
Child (F)			decreased	TOF	+		-	

b) Familial deletions (confirmed with molecular diagnosis) (cont.)

Author Patient no. (sex)	Diagnosis	Thymus	T-cell level	Cardiac malformation	Hypocal-cemia	Mental retardation	Facial dysmorphy	Other features
Ravnan et al. (1994)								
Mother	VCFS							
Child	VCFS							
Mother	VCFS							
Child	VCFS							
Puder et al. (1994)								
Mother	Normal							
Child (F)		small		IAA/VSD/PDA				
Hajianpour et al. (1994)								
Family 1								
Mother				-		+	+	Micrognathia
Proband (M)				-		+	+	High arched palate, micrognathia, fingers hypermobility
Brother (M)				-	seizures	+	+	High arched palate, fingers hypermobility, sacral dimple, syndactyly
Family 2								
Mother				-	+	+	+	Micrognathia, high-arched palate, jejunal atresia
Proband (F)				+		ND	+	Micrognathia, sacral dimple
Sister (F)				-		+	+	

c) Familial deletions (clinical suspicion)(adapted from McLean et al., 1993)

	Diagnosis	Thymus	T-cell level	Cardiac malformations	Hypocal-cemia	Mental retardation	Facial dysmorphy	Other features
Strong (1968)								
Mother				RAA			+	Cleft palate, learning disability
Daughter	VCFS			RAA			+	Cleft palate, learning disability
Daughter	VCFS			RAA			+	Cleft palate, learning disability
Son	VCFS			RAA			+	Cleft palate, learning disability, syndactyly
Steele et al. (1972)								
Mother		small	decreased		+			
Proband	DGS	absent	normal		+		+	
Half-brother	DGS	absent			+			
Young et al. (1980)								
Mother				None		+	+	Cleft palate, learning disability
Daughter	VCFS			VSD		+	+	Cleft palate, learning disability
Mother				None		+	+	Sm cleft palate, learning disability
Daughter	VCFS			VSD		+	+	Cleft palate, learning disability
Mother				None		+	+	Cleft palate, learning disability
Daughter	VCFS			TOF		+	+	Cleft palate, learning disability
Son	VCFS			TOF		+	+	Cleft palate, learning disability
Mother				None		+	+	Cleft palate, learning disability
Son	VCFS			Cardiopathy		+	+	Cleft palate, learning disability
Shprintzen et al. (1981)								
Family 1								
Mother	VCFS			None			+	Cleft palate, learning disability
Child (F)	VCFS			+			+	Cleft palate, learning disability
Family 2								
Mother	VCFS			None			+	Cleft palate, learning disability
Child (F)	VCFS			+			+	Cleft palate, learning disability
Family 3								
Mother	VCFS			None			+	Cleft palate, learning disability
Child 1 (F)	VCFS			+			+	Cleft palate, learning disability
Child 2 (M)	VCFS			+			+	Cleft palate, learning disability

c) Familial deletions (clinical suspicion) (cont.)

	Diagnosis	Thymus	T-cell level	Cardiac malformations	Hypocal-cemia	Mental retardation	Facial dysmorphy	Other features
Shprintzen et al. (1981) cont.								
Family 4								
Mother	VCFS			None			+	Cleft palate, learning disability
Child (M)	VCFS			+			+	Cleft palate, learning disability
Lagrue and Griscelli (1984)								
Mother								Cleft palate, microretrognathia
Child (F)	DGS							
Child (F)	DGS							
Williams et al. (1985)								
Father				None		+	+	Cleft palate, learning disability
Son	VCFS			None			+	Sm cleft palate, bifid uvula, learning disability
Wraith et al. (1985)								
Mother				TOF			+	Sm cleft palate, learning disability
Son	VCFS			TOF			+	Holoprosencephaly.
Meinecke et al. (1986)								
Mother (#2)							+	
Son (#1)	VCFS			VSD		+	+	Hypernasal speech
Mother (#7)				None		+	+	Learning disability
Daughter (#5)	VCFS			PDA, PA		+	+	Learning disability
Son (#6)	VCFS			None		+	+	Learning disability
Stevens et al. (1990)								
Father				None			+	Learning disability, Sm cleft palate
Son	VCFS			VSD, TA, ASD, IAA				
Lipson et al. (1991)								
Mother				+			ND	
Son (proband)	VCFS			+		+	+	Learning disability, hypernasal speech
Son (twin)	VCFS			+			ND	ND

d) Inherited unbalanced translocations

	Diagnosis	Thymus	T-cell level	Cardiac malformations	Hypocal-cemia	Mental retardation	Facial dysmorphy	Other features
Dallapicolla et al. (1989)								
45,X, -22, t(X;22)(q28;q11) de novo, X and 22 **pat**	DGS	Absent	Decreased	TA, RAA LSA	-		+	
El-Fouly et al. (1991)	DGS							
46,XX, -22,+ der9, t(9;22) (q21.13;q11.2) **mat**		Hypoplastic	ND	IAA, TA, VSD	-		+	
Bowen et al. (1986)	DGS							
45,XY, -22, t(18;22) (q12;q11.2) **pat**		Absent	Decreased	VSD	-		+	High palate
Kelley et al. (1982)								
45,XX, -22, t(10;22) (q26;q11.2) **pat**	DGS	Hypoplastic	Decreased	TA, VSD	+		+	Bifid uvula
45,XY, -22, t(20;22) (q11;q11.2) **mat**	DGS	Hypoplastic	ND	TGA, VSD RSA, PA	+		+	Dysplastic kidneys
Pivnick et al. (1990)	DGS							
46 XX, -22, + der(9) t(9;22) (q22;q11.2) **mat**		Absent (X rays)	Decreased	IAA, VSD PDA			+	Cleft lip and palate
de la Chapelle et al. (1981)	DGS							
45,XX, -22, t(20;22) (q11;q11.2) **pat**								
child III12 proband		Absent		AAA, VSD			+	Cleft palate
child III17 (M)		Absent		AAA, VSD TA				
child III18 (F)		Absent		AAA, VSD			+	Cleft palate
45,XX, -22, t(20;22) **mat**								
child III23 (M)		Absent	Decreased	TGA, VSD			+	Cleft lip and palate
Greenberg et al.(1984)	DGS							
GM 5401 : 45,XY,-4,-22, +der(4) t(4;22)(q35.2;q11.2) **mat**		ND	Decreased	TA	+	+	+	None

CHAPTER 4

4.1 Introductory comment

In order to identify the molecular defect underlying DGS, we aimed at cloning the breakpoint of a balanced t(2;22)(q14.1;q11.1) translocation associated with a DGS phenotype. The first step was to construct a detailed physical map of the DGS critical region and the results of this endeavour are presented in this section. Probes were obtained from various sources and were mapped on chromosome 22 using an extended chromosome 22 hybrid panel. Thirty microsatellite markers obtained form Dr. J. Weissenbach (Généthon, France) were localized by PCR amplification of DNA from the hybrids. Ten other cosmid loci were assigned to chromosome 22 and finely mapped with respect to the DGS deleted region using one-color FISH on DGS patients bearing chromosomal translocations and two-color FISH to order probes relative to one another.

4.2 Physical mapping of 30 CA-repeats on human chromosome 22.

Abstract

We report the physical mapping of 30 microsatellite markers specific for chromosome 22, by PCR amplification of DNA from hybrids which divide the long arm in 29 subregions. This work permits to refine further the genetic linkage ordering previously published.

In the recent past, there has been considerable effort in generating polymorphic markers specific for a given chromosome and ordering them by genetic linkage analysis. However, it remains important to link these polymorphic markers to physical anchors on the chromosome; first, to be able to integrate together physical and genetic maps and second, to permit the characterization of genes responsible for diseases associated with chromosomal rearrangements, either constitutional or acquired. To this end, we have precisely localized 30 microsatellite markers on chromosome 22 using a panel of somatic cell hybrids that divides the long arm of chromosome 22 in 29 subregions (Delattre et al., in preparation).

Sublocalization of 36 CA-repeat loci on chromosome 22 was attempted. A genetic map for 27 of them has been previously published (2) and primer sequences for the remaining 9 are indicated in Table 1. Mapping of a locus was performed by PCR amplification of DNA from hybrid cell lines containing different parts of chromosome 22 (Delattre et al., in preparation). The observation of an amplification product was indicative of the presence of this locus in the part of chromosome 22 contained in the hybrid. PCR was performed in a volume of 10 µl, with 25 ng of hybrid DNA, in a mixture containing 1.5 mM $MgCl_2$, 20 mM dNTP, 1X Perkin-Elmer Cetus buffer, 0.3 mM of each of the primers and 0.25 unit of Taq polymerase. The PCR conditions consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec and elongation at 72°C for 1 min, and a final elongation at 72°C for 5 min. Controls included human genomic DNA, mouse and hamster DNAs and PCR mix without template. The amplification products were visualized on 1.5% agarose gels.

Thirty CA-repeat markers were successfully localized to subregions of chromosome 22 (Fig.1). The remaining 6 did not give an interpretable pattern on the hybrid panel or gave amplification products similar in size between human and hamster or mouse loci.

In some occurrences, the hybrid breakpoints permitted a more precise ordering of the microsatellite markers than the previously published genetic map (2). Thus, D22S424 is clearly distal to

D22S280 and D22S422, and D22S277 is proximal to D22S278 (Fig.1). In addition, D22S279 is distal to D22S423, D22S284 and D22S428; D22S418 is distal to these 4 markers and D22S282 is distal to D22S418 and D22S276. Furthermore, our mapping data definitively confirm the proximal-distal order of D22S425 and D22S446.

In previous studies, a lack of markers in the proximal and subtelomeric parts of chromosome 22 has been reported (1, 3). From our map, there appears to be indeed an uneven distribution of the markers within the different panel subregions. Three areas seem to display a low density of marker loci: the subcentromeric (group 1) and subtelomeric (groups 13-14) regions and the area covered by subregions 6 to 9. However, this uneven distribution of the markers could be related to the difficulty in estimating the lengths of these regions, since few of the hybrid breakpoints are precisely mapped relative to chromosomal bands. For the same reasons, the correlation between G-bands and high density of CA-repeat loci remains approximate.

Therefore, mapping of these microsatellite markers within 29 subregions of chromosome 22 covering on average 1 Mb of genomic DNA each, will permit to approach efficiently the cloning of genetic disorders mapped to this chromosome.

Acknowledgements

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References

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Figure 1

Physical localization of the microsatellite markers on chromosome 22. An ideogram of chromosome 22 is shown on the left. The shaded bars represent the part of chromosome 22 remaining in each hybrid cell lines and the vertical lines the bins defined by the hybrid breakpoints. The position of each microsatellite is indicated between the vertical lines delimited by the hybrids.

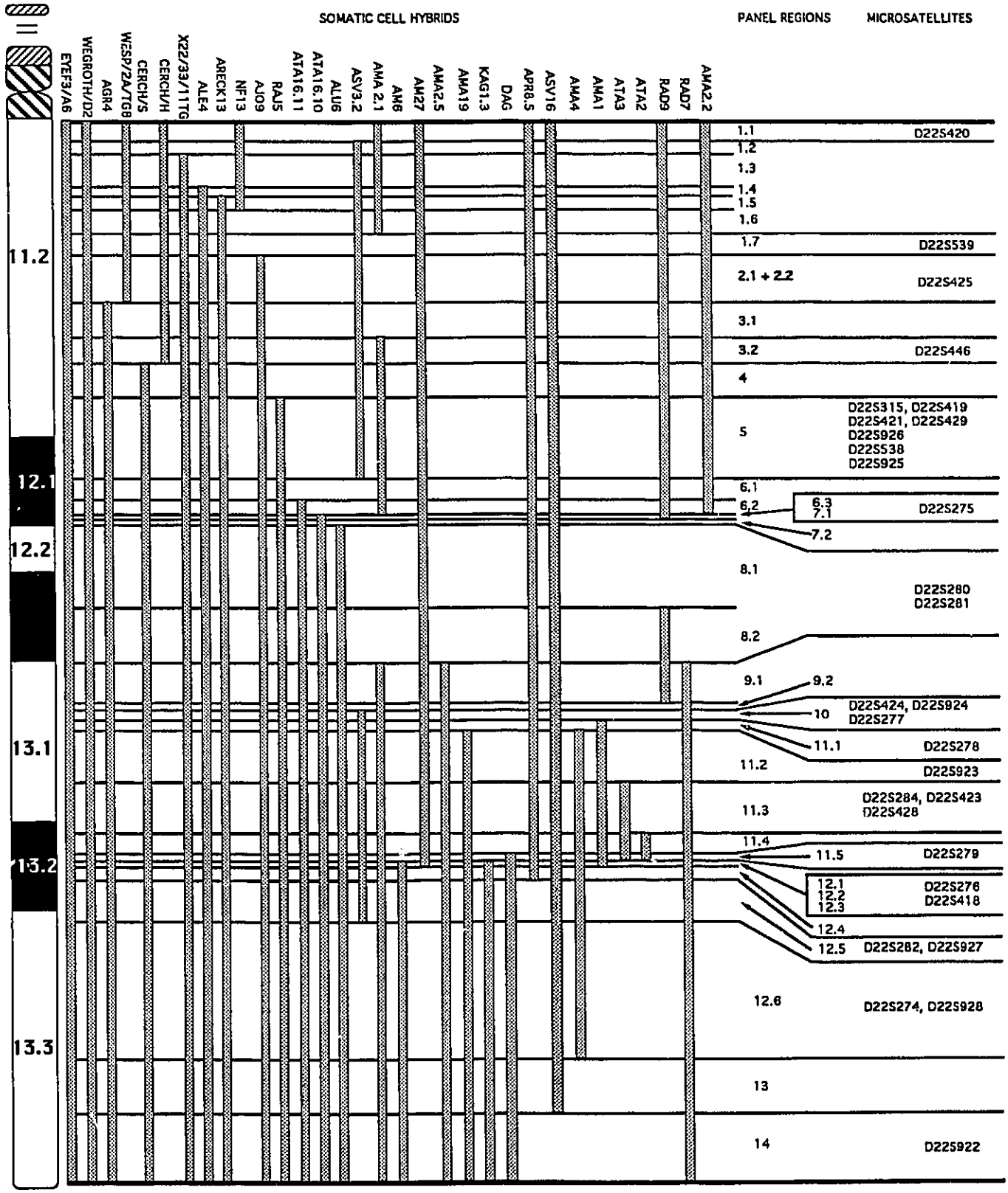

SOMATIC CELL HYBRIDS
PANEL REGIONS
MICROSATELLITES
EYEF3/A6
WEGROTH/D2
AGR4
WESP/2A/TG8
CERCH/S
CERCH/H
X22/33/11TG
ALE4
ARECK13
NF13
AJ09
RAJ5
ATA16.11
ATA16.10
ALU6
ASV3.2
AMA 2.1
AM6
AM27
AMA2.5
AMA19
KAG1.3
DAG
APR8.5
ASV16
AMA4
AMA1
ATA3
ATA2
RAD9
RAD7
AMA2.2
11.2
12.1
12.2
13.1
13.2
13.3
1.1
1.2
1.3
1.4
1.5
1.6
1.7
2.1 + 2.2
3.1
3.2
4
5
6.1
6.2
6.3
7.1
7.2
8.1
8.2
9.1
9.2
10
11.1
11.2
11.3
11.4
11.5
12.1
12.2
12.3
12.4
12.5
12.6
13
14
D22S420
D22S539
D22S425
D22S446
D22S315, D22S419
D22S421, D22S429
D22S926
D22S538
D22S925
D22S275
D22S280
D22S281
D22S424, D22S924
D22S277
D22S278
D22S923
D22S284, D22S423
D22S428
D22S279
D22S276
D22S418
D22S282, D22S927
D22S274, D22S928
D22S922

Table 1

Locus name and primer sequence for the 9 unpublished microsatellite markers.

Marker name	Locus symbol	CA strand primer	GT strand primer
AFM031xc11	D22S922	TATCTTGATGGTGGTGTTGG	TTCCTCAGTTTTACCTGTGCT
AFM234zh4	D22S923	TTCAGTTGATTTATTGGGTATTTTA	GCCTGCAAACTTGGTAATC
AFM248xd1	D22S924	GGCCTACTGCATGTCCC	AATGAGCCACCGGGAG
AFM291ve5	D22S926	GCCGGCAATTTCTAATAAAC	GGTGGGTCAAACCAGA
AFM320yg5	D22S927	CTGCTGAGAGTTTCATGGG	CTTTCTTGGGCAAAGACG
AFM316wd5	D22S538	CATTTTACCTCTTGACCCAG	CGAATACAGAAGATTCCCTCT
AFMa037zd1	D22S539	CATTATGGCTGTAGGCTGTA	CATACCCAATGCAATATGAA
AFMa134yf9	D22S925	CTGGCCCCCATCCAAC	GCGAGTGATTTCTTATTTCCTGCTT
AFMa048wa5	D22S928	TGCAAAGTGCTGGAGG	TGAAGATGGCTAGTACGGG

4.3 Appendix 2

Unpublished data on the physical map of the DGS deleted region

During the course of this research project, I have been involved in the mapping of a number of probes from various sources by FISH, the aim being to increase the number of loci mapped in the DGS region. I have mapped 10 Not I linking clones isolated in the laboratory (Sanson et al., 1992). Three were on the proximal part of chromosome 22, one within the DGCR, and the two others flanking proximally and distally the region, respectively. Three were localized on the telomeric part of chromosome 22 (22q13-qter) and 4 were mapped on other chromosomes.

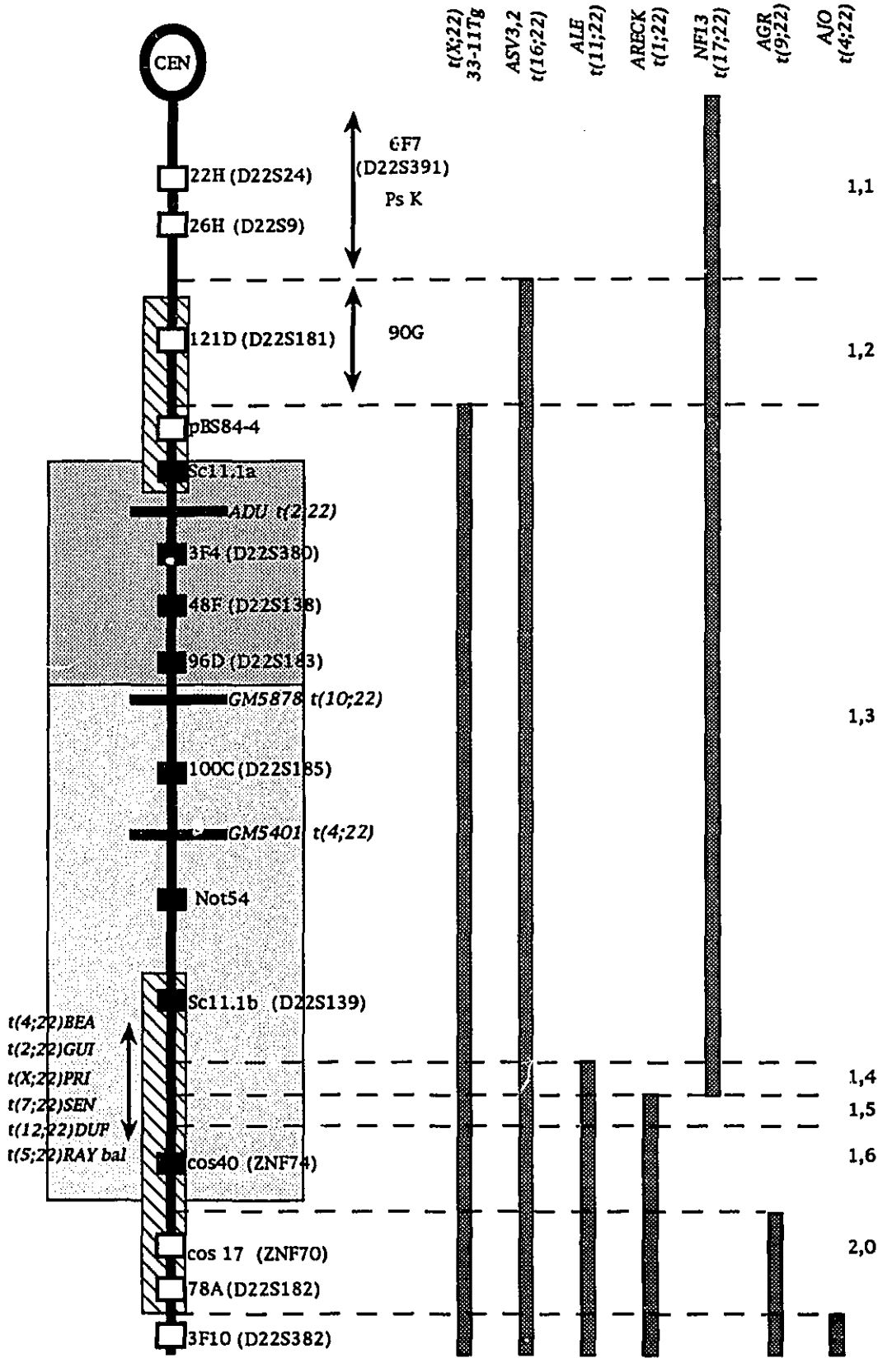
Four cosmids were isolated by virtue of low stringency hybridization to a homeobox-containing probe, but ended up not containing an homeotic sequence. Two of these cosmids recognize loci mapping to chromosome 22 (22q11.2, proximal to the DGS deleted region, and 22q13.3) and 2 mapped on other chromosomes (1q33-41, 12q23).

Five single-copy probes (D22S181-S185) already assigned to the proximal part of chromosome 22 were localized more finely using a chromosome 22 hybrid panel (Van Biezen et al., 1993). Because they all mapped either in the DGS deleted region, or immediately flanked it, cosmids were isolated from these probes, and were used in FISH experiments to map these loci with respect to translocation breakpoints, or in two-color FISH experiments for distance estimation.

Finally, a number of other cloned sequences were mapped, but were of limited usefulness in the building of the DGS region physical map. The latest version of this physical map, work begun by Desmaze et al. (1993a) is presented in the accompanying figure.

The vertical line represents chromosome 22 and the array of probes mapped in the DGS region are depicted by squares (open squares: conserved in DGS; filled squares: deleted in DGS. The horizontal lines represent translocation breakpoints associated with

DGS phenotypes, whereas, on the right side of the panel the shaded rectangles delimitate the region of chromosome 22 present in somatic hybrids. The commonly deleted region is represented by the light-shaded rectangle and includes the minimally deleted region (dark-shaded rectangle). This commonly deleted region has been estimated to be 2-3 Mb long. The critical region extends, distal to proximal, from the breakpoint of the t(10;22) translocation to locus pBS84-4. A number of moderately repeated sequences have been localized on either side of the commonly deleted region, and these are represented by stipled rectangles.


CEN
22H (D22S24)
26H (D22S9)
6F7
(D22S391)
Ps K
121D (D22S181)
90G
pBS84-4
Sc11.1a
ADU t(2;22)
3F4 (D22S380)
48F (D22S138)
96D (D22S183)
GM5878 t(10;22)
100C (D22S185)
GM5401 t(4;22)
Not54
Sc11.1b (D22S139)
t(4;22)BEA
t(2;22)GUI
t(X;22)PRI
t(7;22)SEN
t(12;22)DUF
t(5;22)RAY bal
cos40 (ZNF74)
cos 17 (ZNF70)
78A (D22S182)
3F10 (D22S382)
t(X;22) 33-11Tg
ASV3,2 t(16;22)
ALE t(11;22)
ARECK t(1;22)
NF13 t(17;22)
AGR t(9;22)
AJO t(4;22)
1,1
1,2
1,3
1,4
1,5
1,6
2,0

CHAPTER 5

5.1 Introductory comment

The distance between the loci flanking the balanced t(2;22) translocation breakpoint was estimated using two-color FISH, and a relationship involving percentage of overlapping fluorescent spots and genomic distance. This distance was estimated to be between 100 and 300 kb. A chromosome walk was undertaken from loci flanking distally the balanced translocation breakpoint. Two cosmid contigs were constructed: the 48F8 contig, 170 kb and the 3F4 contig, 325 kb. The 3F4 contig spans the balanced translocation breakpoint, and a novel gene, potentially encoding for an adhesion receptor, was isolated with a phylogenetically conserved sequence and mapped within the contig.

5.2 Cloning of a balanced translocation breakpoint in the DiGeorge syndrome critical region and isolation of a novel potential adhesion receptor gene in its vicinity.

Abstract

Deletions of the 22q11.2 have been associated with a wide range of developmental defect (notably DiGeorge syndrome, Velo-Cardio-Facial syndrome, conotruncal anomaly face syndrome and isolated conotruncal cardiac defects) classified under the acronym CATCH 22. A DiGeorge syndrome patient bearing a balanced translocation whose breakpoint maps within the critical region has been previously described. We report the construction of a cosmid contig spanning the translocation breakpoint and the isolation of a gene mapping 10 kb telomeric to the breakpoint. This gene encodes for a novel putative adhesion receptor protein, which could play a role in neural crest cells migration, a process which has been proposed to be altered in DiGeorge syndrome.

Introduction

DiGeorge syndrome (DGS) is a developmental defect which associates hypo- or aplasia of the thymus and parathyroids, conotruncal cardiac malformations and typical facial features (1). Because these structures are partially derived from the third and fourth pharyngeal pouches, it has been suggested that DGS is the consequence of a defect in their development during the 4th to 7th week of gestation, possibly in their interaction with the cephalic neural crest cells (2). A great majority of DGS cases is associated with monosomy for the 22q11.2 chromosomal region either through a large interstitial deletion (at least 2 Mb) of that region (inherited or de novo) or through an unbalanced translocation involving chromosome 22 (reviewed in 3).

More recently, deletions involving the same chromosomal region as DGS have been reported for other closely related syndromes such as the velo-cardio-facial syndrome (VCFS) (4-6), isolated conotruncal cardiac defects (either sporadic or familial) (7, 8), the CHARGE association (9, 10) and the conotruncal anomaly face syndrome (11), which prompted the coining of the acronym CATCH 22 (Cardiac defect, Abnormal facies, Thymic hypoplasia, Cleft palate, Hypocalcaemia, chromosome 22q11 deletions) to designate this group of related syndromes (12). The CATCH 22 group of syndromes appears to be a major cause of birth defects with a minimum prevalence estimated to 1 in 5000 (13). It has been proposed that deletions in 22q11.2 may be etiological in 5% of all newborns with heart defects (14). It thus appears that there is/are important gene(s) playing a role during embryogenesis in this chromosomal region.

While DGS was originally thought to be a contiguous gene syndrome (15, 16), it has not been possible to correlate the extent of 22q11.2 deletions with the presence or the severity of a phenotypic feature (17-19). Furthermore, a wide variability in the phenotype has been observed within CATCH 22 families, indicating again that the severity of the disorder is not strictly related to the extent of the deletion. In addition, one single observation of a daughter and her

mother both bearing a balanced t(2;22) (q14.1;q11.1) translocation and displaying DGS/VCFS phenotypes (herein referred to as ADU and VDU) has been reported (20). All these observations lead us to propose that one major gene may be involved in the pathogenesis of the CATCH 22 syndromes, and that the ADU translocation breakpoint may interrupt this locus. In a few patients, neither deletions nor chromosomal aberrations are observed (17, 21) suggesting that these cases could be due to very small deletions or point mutations within the major CATCH 22 gene. These cases could become essential in confirming the implication of a candidate gene in the etiology of CATCH 22.

Considerable efforts have been made, in the recent past, to build a detailed physical map of the 22q11.2 region by localizing probes with respect to translocation breakpoints or deletion borders associated with CATCH 22 cases, in order to define a minimal critical region (DGCR) (13, 18, 22). The critical region was never smaller than 300 kb (13), extending centromeric to telomeric from the breakpoint of the GM00980 cell line [derived from a VCFS patient having the following karyotype: 45, XX, -11, -22, +der(11), t(11;22) (q25;q11)] to the pH11 locus described in (18), and included the ADU/VDU balanced translocation breakpoint. Up to now, only one gene has been cloned from the DGCR (13). This gene, TUPLE1, contains WD40 domains and shows a significant homology to the Saccharomyces cerevisiae Tup1 transcriptional regulator gene. However, TUPLE 1 maps about 100kb telomeric to the ADU breakpoint (Scambler P.J., oral communication, 26th meeting of the European Society of Human Genetics, 1994).

With the aim of identifying the molecular defect underlying the CATCH 22 group of syndromes, we have cloned the ADU balanced translocation breakpoint by chromosome walking. Furthermore, we report the isolation of a gene encoding for a potential adhesion receptor protein. This gene does not encompass the breakpoint, but its 3' end maps only 10 kb telomeric to the balanced translocation. We propose a mechanism by which this gene could be involved in the pathogenesis of CATCH 22.

Materials and Methods

Cell lines, probes and libraries

ADU is a cell line established from lymphocytes of a patient bearing a balanced t(2;22) (q14.1;q11.1). The patient had an aortic coarctation, mild reduction in T cells, a transient mild hypocalcemia during the neonatal period and a facial dysmorphism. The mother, who bears the same translocation (cell line VDU) has an hypernasal voice, micrognatia and an inverted T4/T8 ratio. The GM05878 cell line has been established from the father of a DGS patient with an unbalanced t(10;22) (q26;q11.2) (45). Its position within the DGS critical region has been characterized in (22, 46).

Cosmid 3F4 (D22S380) has been isolated from a Not I linking clone library (47). Probe Sc11.1 is described in (13).

The chromosome 22-enriched genomic cosmid libraries (LL22NC01 and LL22NC03) have been constructed in the Lawrence Livermore National Laboratory. The human fetal brain cDNA library cloned in the λZAP II vector (cloning sites EcoRI) has been purchased from Stratagene (LaJolla, CA).

FISH

Cytogenetic preparations and FISH were performed as described in (22).

Northern and Southern analysis

Multiple adult and fetal tissues Northern blots were purchased from Clontech (Palo Alto, CA.) and were probed according to manufacturer's instructions. Washes were done in 0.1SSC/0.1% SDS at 65°C.

DNA was extracted from cultured human cell lines and blood samples from human, rat and mouse, digested with restriction enzymes, migrated on a 0.8% agarose gel and transferred on Hybond N+ nylon membrane. Hybridizations were performed at 50°C. For pulsed-field gel electrophoresis, DNA from the DGS cases and control individuals was prepared into plugs in low-melting point agarose, digested with rare-cutting restriction enzymes, and electrophoresed

under various conditions of pulse, voltage and migration time. The gels were then blotted and hybridized with probes from the region. Washes were in 1XSSC/0.1 %SDS at 60°C for "zoo blots" and in 0.1SSC/0.1 % SDS at 65°C for human genomic blots. If a band on mouse or rat DNA was visible, the blots were rewashed at increasing stringency.

Sequencing and database searches

Lambda cDNA clones were subcloned in plasmids using the λZAPII plasmid rescue procedure according to the manufacturer's specifications (Stratagene, La Jolla, CA). Clone BF1-2EN was shotgunned in the M13 vector and sequenced by the dye primer methodology using an Applied Biosystem ABI 373A fluorescent sequencer. Clone BF1-05EB was sequenced by primer walking using the dideoxy chain terminator method. Assembly of DNA sequences was performed using the computer program "Autoassembler" (Applied Biosystems, Branchburg, NJ). The sequences were used to search the Swissprot and Genbank databases using BLAST and FASTA, available on the BISANCE server (CITI 2, Paris, France).

Results

Cloning of the ADU breakpoint

Cosmid probe 3F4 recognizes a locus mapping telomeric to the chromosome 22 ADU breakpoint, but centromeric to the GM05878 one (the latter lies telomeric to the GM00980 breakpoint). Two-color fluorescence in situ hybridization (FISH) was used to estimate its distance from a locus flanking proximally the ADU breakpoint (Sc11.1prox, gift from P. Scambler) and lying within the DGCR. The distance between the 3F4 and Sc11.1prox loci was estimated to be between 100 and 300 kb, by assessing the percentage of overlapping fluorescent spots and using a relationship derived in (23). Therefrom, we undertook a chromosome walk by constructing a contig from both extremities of cosmid 3F4. The centromere-telomere orientation of the contig was determined by hybridization of single-copy probes from the region on pulsed-field gel electrophoresis blots. A probe from one side of the contig hybridizes to the same 200 kb MluI band than a probe isolated from a cosmid which corresponding locus has been ordered telomeric to the 3F4 locus by two-color FISH. The contig, covering 325 kb of genomic DNA (Fig. 1), could not be prolonged further than 70 kb toward the telomere although the cosmid libraries used represent 8 genome-equivalents. These data support the previous finding that this region is under-represented in most genomic libraries (13).

After 3 steps (70 kb) toward the centromere, the ADU breakpoint was crossed, as shown by FISH on metaphases from the ADU patient with 2 cosmids from the contig (Fig. 2). The crossing of the breakpoint was confirmed and fine-mapped by Southern hybridization on ADU's DNA (Fig. 3a-b). Both ADU and VDU display the same extra bands, therefore suggesting they bear the same rearrangement (Fig. 3c-d).

Isolation of coding sequences around the ADU breakpoint

A systematic search for phylogenetically conserved sequences was performed in a region of 55 kb centromeric and 30 kb telomeric to the breakpoint. A 0.8 kb StuI-StuI fragment from the 130F cosmid

recognizes a sequence conserved in rodent DNA, even at high wash stringency (0.1SSC/0.1% SDS, 65°C) and was used to screen a human fetal brain cDNA library. Several clones were obtained and assembled into a composite cDNA (Fig. 4a) which turned out to be 4.4 kb long. Two clones (BF1-2EN, 3.6 kb and BF1-05EB, 870 bp), spanning the entire gene and overlapping over 86 bp were completely sequenced and shown to contain an open reading frame with an initiator methionine and a 3' poly-A tail (Fig 4b).

Both cDNA clones hybridize to the same 4.4 kb transcript on Northern blots prepared from poly-A+ RNAs from multiple fetal and adult tissues (Fig. 5). The transcript recognized by these cDNA probes is expressed in all tissues examined, but at variable levels.

Mapping of BF1-2EN/BF1-05EB within the DGCR

Subfragments corresponding to both extremities of the cDNAs were hybridized to the cosmid contig, and the orientation of transcription appears to be from telomere to centromere. In addition, the cDNA was confirmed to map back to the expected region of chromosome 22 using a panel of somatic cell hybrids (Delattre et al., submitted). The gene spans 65 kb of genomic DNA and the 3' end maps 10 kb telomeric to the ADU breakpoint (Fig. 1).

The BF1-2EN/BF1-05EB cDNA encodes a putative adhesion receptor protein

The BF1-2EN/BF1-05EB cDNA contains a 550 amino acid long open reading frame. There is a stop codon at position -1, just before the initiator methionine. The 5' non-coding end is 147 bp long and the 3' one is 2.6 kb, with a potential polyadenylation signal at position 4377 confirmed on 2 different clones (Fig. 4b). A search in nucleotide databases indicated that nucleotides position 2823 to 3171 and 3028 to 3398 correspond to expressed sequence tags IB209 and EST07022, isolated from human cDNA libraries (accession number T03393 and T09129 respectively).

The predicted protein has a molecular weight of 61 kd and is mostly hydrophilic. The first 24 amino acids (residues -24 to -1) have the characteristics of a signal peptide: they form a sequence

that is mainly hydrophobic and that presumably terminates in position -1 and -3 with leucine and glutamic acid (24). The mature protein would then consist of 526 amino acids with a predicted molecular weight of 58 kD. A second stretch of uncharged, mainly hydrophobic, amino acids from position 326 to 344, could be compatible with a membrane-spanning domain.

The N-terminal end of the protein (amino acids 4 to 42) is characterized by a regular pattern of 6 cysteine residues separated by 7 amino acids. This sequence is significantly homologous to the Cys-rich repeats present in the low-density lipoprotein receptor (LDLR) of many species, including human (25). This type of repeated motif is also found in many mammalian proteins associated with the immune system and host defense (human complement C9, human complement factor 1), with lipids metabolism (very low-density lipoprotein receptor, macrophage scavenger receptors, LDL receptor-related protein), and with basement membrane (mouse perlecan, *C. elegans* heparan sulfate proteoglycan or unc-52) (26-30). A comparison of this region of the BF1-2EN protein with the corresponding regions in some of these proteins is shown in Fig. 6.

The LDLR, the best characterized of these proteins, contains 8 such repeats, which are thought to form the ligand-binding domain, especially because of the presence in almost every repeat unit of amino acids with negatively charged side chains (DXSDE, Fig. 6) that could accomodate the binding of the positively charged residues of apoproteins B and E (25). In addition, the cysteine residues appear to be involved in disulfide bonds, which could impart stability to the LDLR. These main features of the LDLR Cys rich repeat are present in the BF1-2EN protein.

Amino acids 91 to 217 form a domain that shows similarity to the C-type lectin domains of human B cell differentiation antigen (Lyb-2), rat liver asialoglycoprotein receptor, and to rat and human cartilage-specific proteoglycan (31-34) (Fig. 7). The C-type lectins are calcium-dependent carbohydrate-binding proteins and some of them are involved in adhesive interactions between cells (35).

The remaining extracellular part of the protein is rich in cysteine residues, which could be important for the protein secondary structure through disulfide bridges formation.

The C-terminal (cytoplasmic) domain of the protein (amino acids 345 to 526) is mostly acidic and proline-rich. Notably, two sequences DPPPPYXA separated by 10 amino acids are observed, but do not correspond to any specific domain when databases are searched. In view of the relatively uncertain homology with proteins or protein families of known function, we propose to label the BF1-2EN protein DiGeorge critical region gene 2 (DGCR2) until further indications about its function are obtained. In accordance to this labeling, TUPLE1 (13) would then be DGCR1.

Discussion

We report the cloning of a balanced t(2;22) translocation breakpoint borne by a DGS patient, and mapping within the DGS critical region. About 150 kb of genomic DNA on either side of the breakpoint has been cloned by the establishment of a cosmid contig. This contig will represent a powerful tool in the identification of the genetic defect underlying CATCH 22.

A systematic search for conserved sequences in the region has allowed the isolation of a cDNA. The predicted membrane-spanning protein has significant similarities to the Cys-rich repeat region of the LDLR and to C-type lectin domains. All these data lead us to propose that the gene encodes a potential adhesion receptor, i.e. a membrane protein that could mediate specific adhesive interactions (36).

Even though DGCR2 is not interrupted by the ADU breakpoint, this gene could represent an attractive candidate for involvement in the pathogenesis of the CATCH 22 phenotype. First, DGCR2 is hemizygous in all CATCH 22 cases which bear a deletion and it maps within the DGS critical region.

Second, it is the gene mapping the closest to the ADU breakpoint ever reported. Its direct involvement in ADU's pathology can be explained by different mechanisms. It is possible that the balanced translocation exerts a position effect on the DGCR2 gene expression, as has been reported for the occurrence of a r(15) and Prader Willi syndrome or for a balanced translocation occurring in an aniridia family (37, 38). In both cases, the breakpoints occur several kilobases away from the putative disease loci. It is also possible that the ADU breakpoint physically separates a regulatory element from the coding sequence it acts upon, for example an enhancer (the reverse has been suggested for the Burkitt's lymphoma translocation), or a putative imprinting control region from the gene(s) it imprints (as proposed for the Prader-Willi chromosomal region) (39, 40). Inasmuch as there appears to be a nonrandom parental origin of the 22q11 deletions in CATCH 22, this latter

hypothesis should not be neglected. The presence of such elements in the vicinity of the ADU breakpoint is currently under investigation.

Finally, analysis of the DGCR2 sequence leads us to propose an interesting mechanism by which this protein could be etiological in the CATCH 22 pathology. The cephalic neural crest cells have been shown to contribute to the process of aorticopulmonary septation and to the morphogenesis of the thymus and parathyroids in quail-chick chimeras (41, 42). Therefore, DGS has been classified as a neurocristopathy, i.e. DGS would arise as a result of a defective migration of the neural crest cells in the branchial arches or defective interaction of the neural crest cells with these structures (2, 43). Glycoproteins at the cell surface (e.g. fibronectin, laminin, proteoglycans, chondroitin) have been shown to either promote or inhibit crest cells migration (reviewed in 44). It is therefore possible that the C-type lectin and Cys-rich potential ligand binding domains of the DGCR2 protein mediate neural crest cells migration in, or interaction with, the branchial arches. Haploinsufficiency for the DGCR2 protein would then lead to altered levels of adhesion receptors with inability to sustain migration of a sufficient number of neural crest cells. This hypothesis remains to be tested, by knocking-out one copy of the DGCR2 gene in mouse, and by searching for point mutations or small rearrangements within the gene in patients displaying no deletions.

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Figure 1

EcoRI map of the 3F4 contig genomic region, showing the cosmid overlap and the position of the ADU breakpoint. Each double-headed arrow represents a cosmid. Equally shown are the positions of the StuI-StuI conserved sequence, of the 5' CpG island and of the genomic region spanned by the cDNA (dotted line).

3F4 CONTIG

Centro. **Telo.**

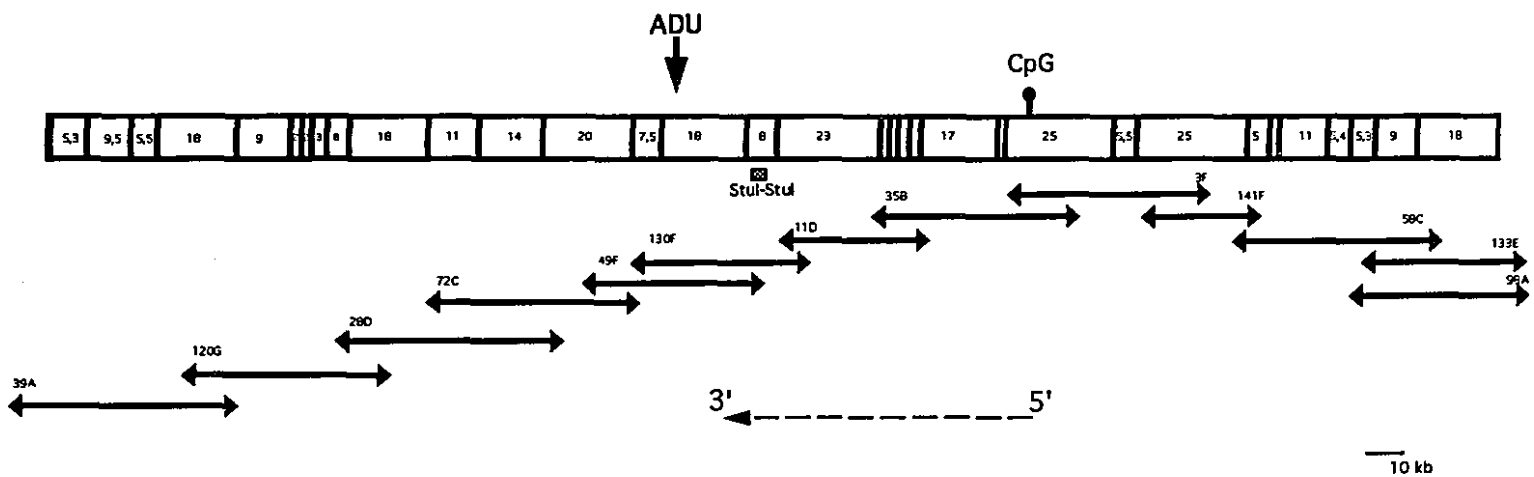


Figure 2

FISH on metaphases from the ADU patient using a) cosmid 3F4. The thin arrow points to the normal chromosome 22 and the thick arrow to the der(2). b) cosmid 72C. Thin arrow: normal chromosome 22; thick arrow: der(22).

a)

b)

Figure 3

Detection of a rearranged band in the ADU and VDU patients. DNA from the ADU and VDU cell lines and control blood sample were digested: Panels a) and b) with EcoRI and probed with two adjacent fragments from the genomic 18 kb EcoRI band: a) 900 bp BssHII-SmaI fragment mapping centromeric to the breakpoint and b) 450 bp SmaI-SmaI fragment mapping telomeric to the breakpoint. Two bands are seen in ADU: the normal locus on chromosome 22 and the 2 derivative chromosomes. Panels c) and d) with XhoI/XbaI and probed with c) same as a); d) same as b). Three bands are observed for each hybridization on ADU and VDU: the relevant locus mapping within the 3F4 contig (5.5 kb band), a secondary locus mapping distal to the DGS deleted region (10 kb band) and the 2 derivative chromosomes.

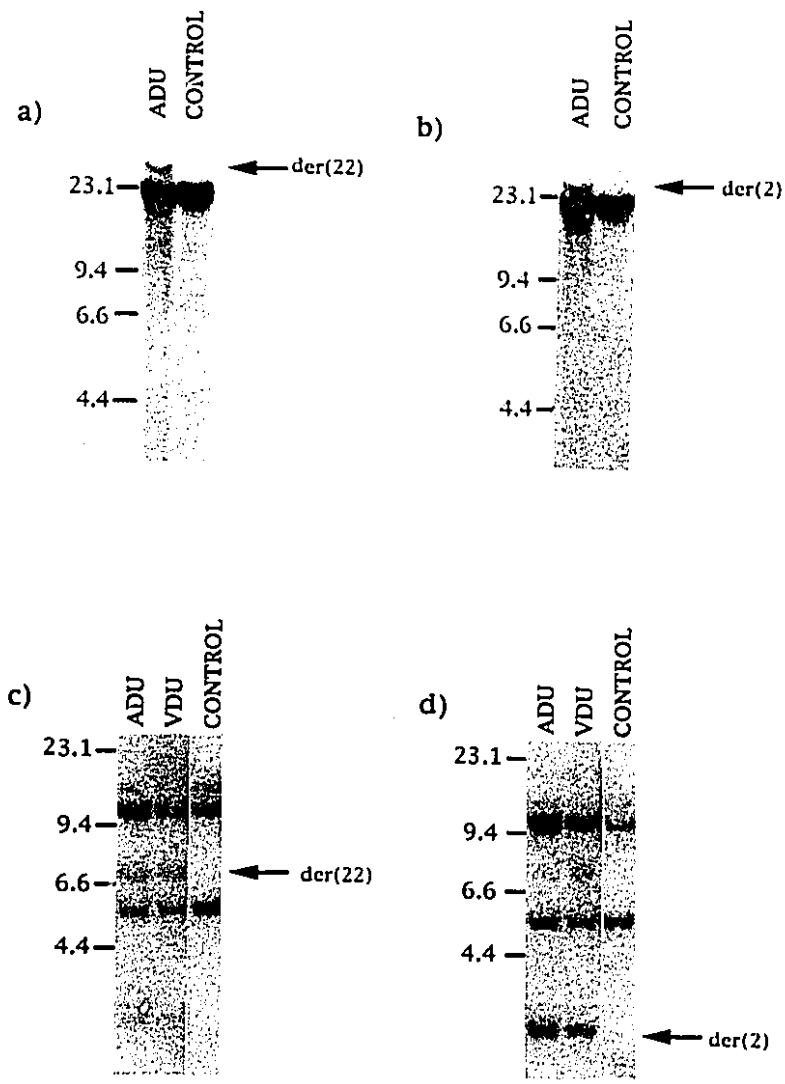
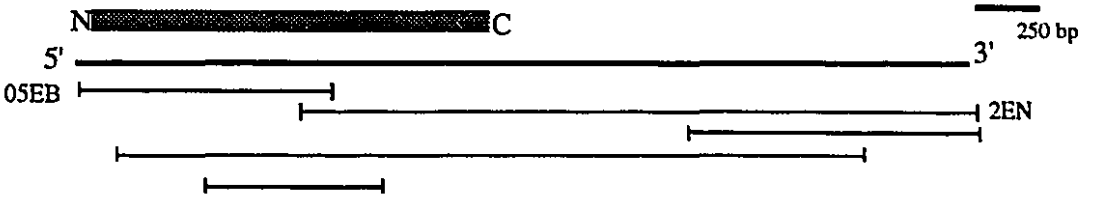

a)
ADU
CONTROL
23.1
9.4
6.6
4.4
der(22)
b)
ADU
CONTROL
23.1
9.4
6.6
4.4
der(2)
c)
ADU
VDU
CONTROL
23.1
9.4
6.6
4.4
der(22)
d)
ADU
VDU
CONTROL
23.1
9.4
6.6
4.4
der(2)

Figure 4

a) Schematic representation of the different cDNA clones obtained, showing the overlap between BF1-2EN and BF1-05EB. The coding region is indicated by the shaded bar.

b) Nucleotide and deduced amino acid sequences The nucleotides sequence is numbered on the right. The first methionine codon is at position 147 with a partial Kozak consensus sequence (TAAATGG) and a stop codon lying immediately before it. The putative signal peptide sequence (amino acids -1 to -24) is underlined. The putative transmembrane domain (amino acids 325 to 344) is in bold characters. The polyadenylation signal (AATAAA) starting at position 4377 is underlined.

A)



B)

```
cgttttctctcctctgtgttggtcgcctgtctcgctcccctctcatgctctctttctctcctctlattttgtctctcactattcgtgttttgtgtttgttttctaggtttggctcaattgctg      120

ccccggggacgatgaacggaggataaATGGTGCCCAAGGCAGACAGCGGCGCCTTCCTGCTGCTCTTCCTGCTCGTGCTCACTGTCACCGAGCCGCTGCCGCCAGAGCTGCGGTGCAACC      240
                           M  V  P  K  A  D  S  G  A  F  L  L  L  F  L  L  V  L  T  V  T  E  P  L  R  P  E  L  R  C  N  P
                          -24                                                                                -1                8

CTGGGCAGTTTGCGTGTCGCAGCGGCACCATCCAGTGCATCCCCCTCCCCTGGCAGTGTGACGGCTGGGCGACTTGCGAGGATGAGAGCGACGAAGCCAACTGTCCAGAAGTGACCGGGG      360
  G  Q  F  A  C  R  S  G  T  I  Q  C  I  P  L  P  W  Q  C  D  G  W  A  T  C  E  D  E  S  D  E  A  N  C  P  E  V  T  G  E
                        18                    28                    38                    48

AGGTGCGTCCTCATCATGGGAAGGAGGCTGTGCATCCGCGGCAGGGGCGGGGCCAGAGGAGGCGACCCTTCGCACTTCCACGCGGTGA-CGTGCGCGCAGCCCGTTCGCTTCAGCAGTTTCC      480
  V  R  P  H  H  G  K  E  A  V  D  P  R  Q  G  R  A  R  G  G  D  P  S  H  F  H  A  V  N  V  A  Q  P  V  R  F  S  S  F  L
                        58                    68                    78                    88

TAGGGAAGTGCCCGACAGGGTGGCACCACTACGAAGGCACGGCCAGCTGCTACCGGGTCTACCTGAGCGGGGAGAACTACTGGGATGCCCGCGCAGACCTGCCAGCGCCTGAATGGCTCTC      600
  G  K  C  P  T  G  W  H  H  Y  E  G  T  A  S  C  Y  R  V  Y  L  S  G  E  N  Y  W  D  A  A  Q  T  C  Q  R  L  N  G  S  L
                        98                   108                   118                   128

TCGCCACCTTCTCCACTGACCAGGAGCTGCGCTTTGTCCTGGCCCAGGAATGGGACCAGCCCGAGCGGAGCTTTGGTTGGAAGGACCAGCGCAAGTTGTGGGTTGGCTATCAGTATGTTA      720
  A  T  F  S  T  D  Q  E  L  R  F  V  L  A  Q  E  W  D  Q  P  E  R  S  F  G  W  K  D  Q  R  K  L  W  V  G  Y  Q  Y  V  I
                        138                   148                   158                   168

TCACTGGCCGGAACCGCTCCTTGGAAGGTCGCTGGGAGGTGGCATTCAAAGGCTCTTCAGAGGTGTTCCTGCCCCAGACCCCATCTTTGCCTCCGCCATGTCTGAGAACGACAACGTGT      840
  T  G  R  N  R  S  L  E  G  R  W  E  V  A  F  K  G  S  S  E  V  F  L  P  P  D  P  I  F  A  S  A  M  S  E  N  D  N  V  F
                        178                   188                   198                   208

TCTGTGCCCAGCTTCAGTGCTTCCATTTCCCCACCCTGCGGCACCACGACCTCCACAGCTGGCACGCCGAGAGCTGCTACGAGAAGTCTTCATTTCTGTGTAAAAGAAGTCAAACATGTG      960
  C  A  Q  L  Q  C  F  H  F  P  T  L  R  H  H  D  L  H  S  W  H  A  E  S  C  Y  E  K  S  S  F  L  C  K  R  S  Q  T  C  V
                        218                   228                   238                   248

TTGACATCAAGGACAACGTGGTGGATGAAGGGTTCTACTTCACCCCTAAGGGGGACGACCCATGCCTGAGCTGCACCTGCCATGGAGGGGAGCCTGAGATGTGTGTGGCTGCTCTCTGTG     1080
  D  I  K  D  N  V  V  D  E  G  F  Y  F  T  P  K  G  D  D  P  C  L  S  C  T  C  H  G  G  E  P  E  M  C  V  A  A  L  C  E
                        258                   268                   278                   288

AGAGGCCCCAGGGCTGCCAACAGTACCGCAAGGACCCCAAAGAGTGCTGCAAGTTCATGTGTCTGGACCCAGATGGCAACAGTCTGTTTGACTCCATGCCCAGCGGGATGCGCCTGGTCG     1200
  R  P  Q  G  C  Q  Q  Y  R  K  D  P  K  E  C  C  K  F  M  C  L  D  P  D  G  N  S  L  F  D  S  M  A  S  G  M  R  L  V  V
                        298                   308                   318                   328

TCAGCTGCATCTCCTCCTTCCTCATCCTGTCACTGGCTGCTCTTCATGGTCCACCGGCTGCGCCAGCGGCGCCGGGAGCGCATCGAGTCCCTGATTGGAGCAAACTTGCACCACTTCAACC     1320
  S  C  I  S  S  F  L  I  L  S  L  L  L  F  M  V  H  R  L  R  Q  R  R  R  E  R  I  E  S  L  I  G  A  N  L  H  H  F  N  L
                        338                   348                   358                   368

TCGGCCGCAGGATCCCTGGCTTTGATTACGGCCCAGACGGGTTTGGCACGGGCCTCACGCCGCTGCATCTTTCTGACGACGGAGAGGGTGGGACTTTCCATTTCCACGACCCTCCACCTC     1440
  G  R  R  I  P  G  F  D  Y  G  P  D  G  F  G  T  G  L  T  P  L  H  L  S  D  D  G  E  G  G  T  F  H  F  H  D  P  P  P  P
                        378                   388                   398                   408

CCTACACGGCATACAAGTACCCGGACATCGGCCAGCCGACGACCCTCCGCCGCCCTACGAGGCCTCCATCCACCCGGACAGTGTGTTCTATGACCCTGCAGACGATGATGCTTTTGAGC     1560
  Y  T  A  Y  K  Y  P  D  I  G  Q  P  D  D  P  P  P  P  Y  E  A  S  I  H  P  D  S  V  F  Y  D  P  A  D  D  D  A  F  E  P
                        418                   428                   438                   448

CTGTGGAGGTCAGCCTGCCAGCCCTGGGGATGGTGGGAGTGAAGGTGCATTACTCCGGCGCCTGGAGCAGCCTCTGCCCACTGCGGGGGCCTCTCTGGCAGACCTGGAAGACTCTGCCG     1680
  V  E  V  S  L  P  A  P  G  D  G  G  S  E  G  A  L  L  R  R  L  E  Q  P  L  P  T  A  G  A  S  L  A  D  L  E  D  S  A  D
                        458                   468                   478                   488

ACAGCAGCAGCGCCCTGCTCGTGCCCCCTGACCCTGCCCAGAGCGGGAGCACCCCAGCTGCAGAGGCACTGCCAGGGGGTGGCCGCCACAGCCGCAGCTCCCTCAATACTGTGGTGTAGA     1800
  S  S  S  A  L  L  V  P  P  D  P  A  Q  S  G  S  T  P  A  A  E  A  L  P  G  G  G  R  H  S  R  S  S  L  N  T  V  V  *
                        498                   508                   518                   526

cggcctggcctgtaccccaacggtctgggagcacctgtctgttgcagaaaacaccggtcccctggggagacttgaaaggccccctgtcccagcctggacgccacgcactgccgcacgtyact     1920
ggcgggctcgcgtgtgtacatagagaccacagccgccttctgccaaaagaagtgatggcctgcaccgagcttccttgaggggcttcagaaacatgcatagcttttggatcactgtcttctc     2040
ctttataaaatggcagaagagtgacaaaattcattcagaccgcacatgttagaggcagggaatgaagaaggtactgtgggccatggccacacctgatgcgttttttggtgggctacttggt     2160
gcagtgtgctgtccagagagacctgctgacccagtctgggacaggcacagtgggagctgccacagtgcccctttgctggccgcctcaggaggggggcctctggaccgtcagtgtggcgtag     2280
gcagtgggtctgcttcagggaggcagcctcttgactttgtcacaacggttgcactgaagatggccccccacaagcccagttgtgaatatcaaggtgaccctgcccctggctgggagctccc     2400
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tctggagccctgggctcttgcctcctgttgaattgcccctgggcctgccccggccccatttgtgccataaagggttgcttcattgcaggaggggtggctgaaaccaccatcctgggctg     2760
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tgtggtgctgctcggggatggactccatgcacagtccacttcttgacacattctcacactgtggctggagacccatttttccatcccatgtggaatgagaatcttgagttgcccacaga     3360
cagacttgaccttgtgccagaactgtccagagctgtgagttttgttactgaattgcctgtccctgagcttggcctgctggagcatgatgctggaggctgtgtgcgggcaggcttgtgct     3480
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aggttccagagcagcaggcatgtgcattatgggtgccaggtggccagtgaggcccgaggaccacgcagcagagtccccccacgggcctgagcctgagagctcagaggattcctgccaggc     3720
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gccacggtttctcagagcccaggtgcccctctgacctgccatgcggaggtgggatttgatactgtacattgtcttgatgcctgttttttttatgttttcattaagggtttttagttttggt     4320
tgggttgacactaacttttcttaagatgctgtgagaatcatttcatccaaatgccaaataaacttgtgttccggaaaa                                              4398
```

Figure 5

Northern blot analysis of DGCR2 in human a) fetal and b) adult tissues. A 4.4 kb transcript is observed in every lanes. Bottom panels show control hybridizations with an actin probe on the corresponding blots. The DGCR2 gene is expressed at low level in fetal liver, at moderate level in fetal brain and at high levels in fetal heart, lungs and kidney. In adult tissues, expression is highest in pancreas, skeletal muscles, lungs, brain and heart, and somewhat lower in kidney and liver. The signal intensity of the actin transcript is equivalent in every lanes, except for adult pancreas.

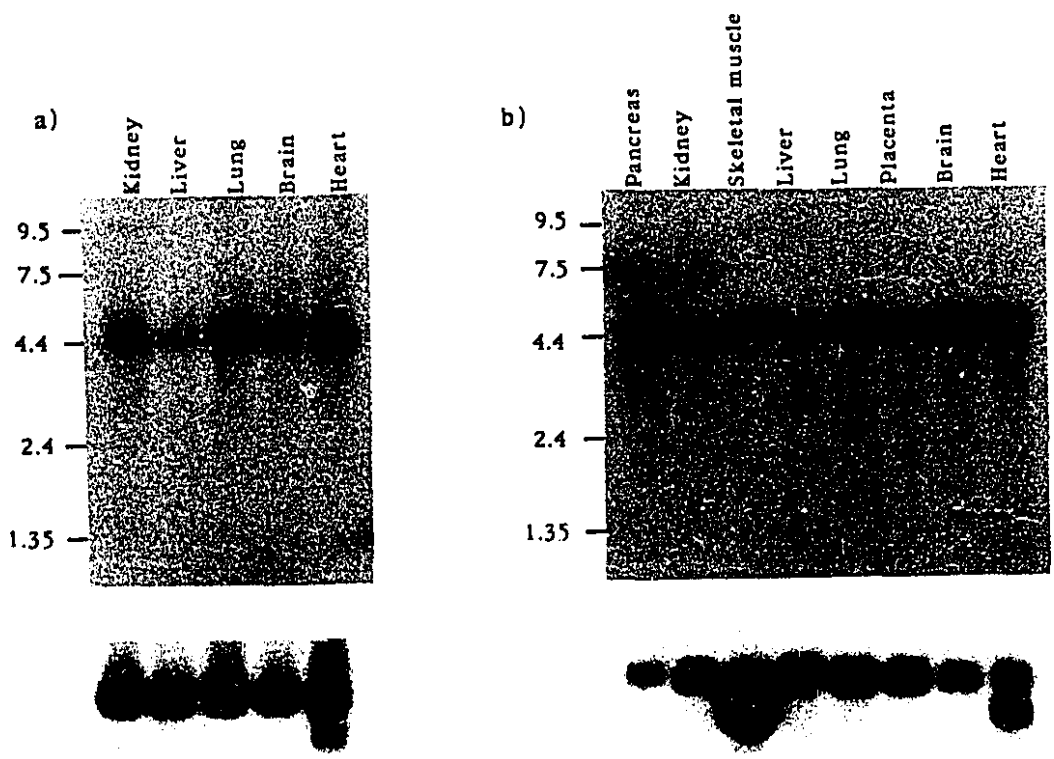


Figure 6

Alignment of the Cys-rich repeat present in the NH_2-terminal region of the DGCR2 protein with the Cys-rich repeat units of other proteins (position in the sequence shown on the left). Homologous residues between repeats are in open boxes. Amino acids that are highly conserved among repeats are shown as a consensus sequence on the bottom line. Amino acids are shown in the single-letter code; Φ stands for aromatic residues. LDLR: low density lipoprotein receptor (25); Comp C9: human complement component C9 (26); Factor 1: human complement control protein factor 1 (27); Unc-52: C. Elegans *unc*-52 gene (28); Perlecan: mouse perlecan (30).

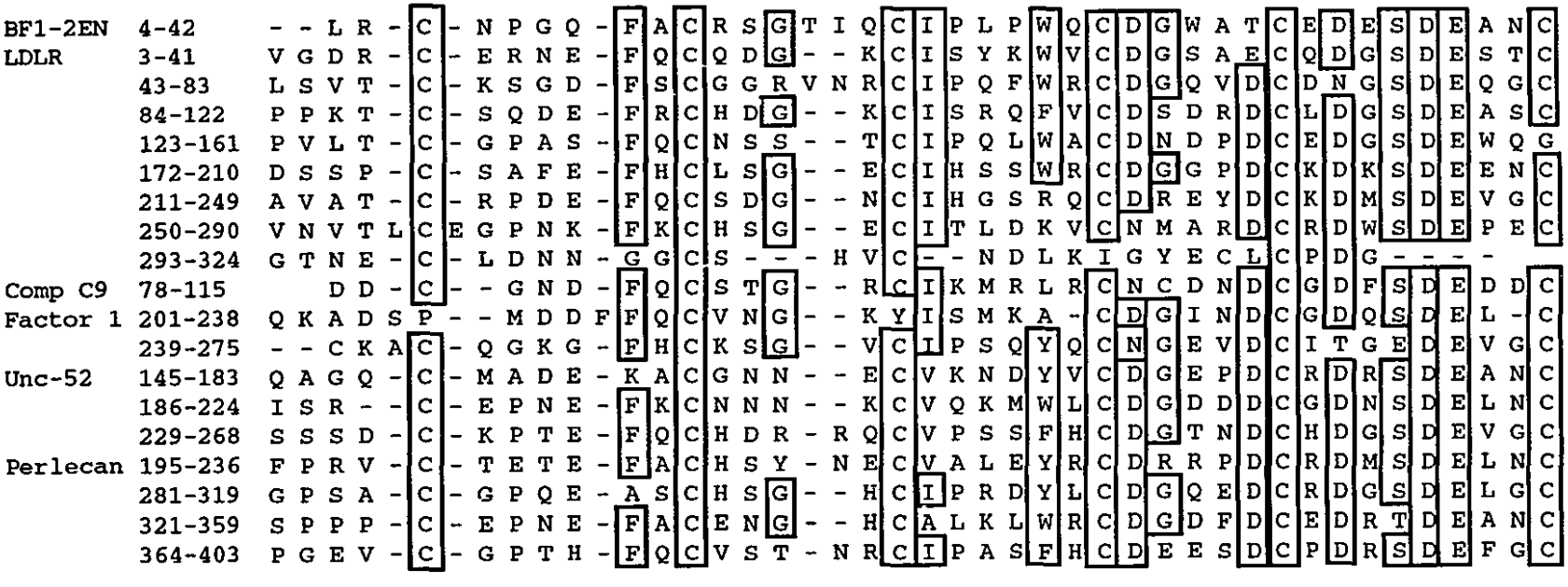
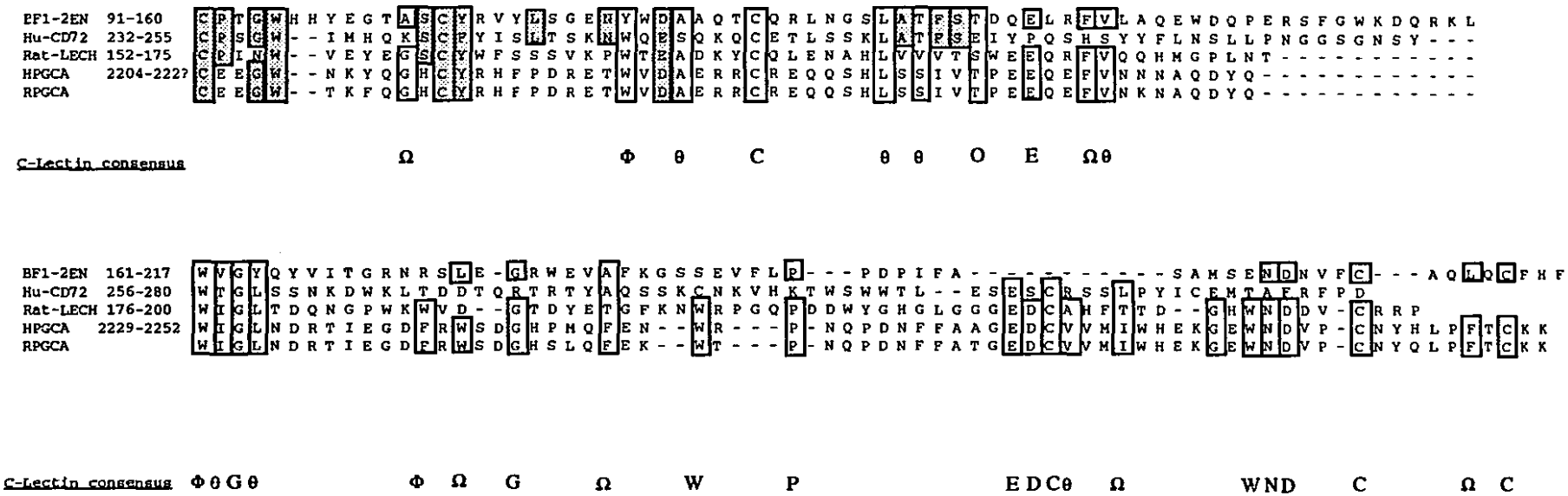

BF1-2EN 4-42 - - L R - C - N P G Q - F A C R S G T I Q C I P L P W Q C D G W A T C E D E S D E A N C
LDLR 3-41 V G D R - C - E R N E - F Q C Q D G - - K C I S Y K W V C D G S A E C Q D G S D E S T C
43-83 L S V T - C - K S G D - F S C G G R V N R C I P Q F W R C D G Q V D C D N G S D E Q G C
84-122 P P K T - C - S Q D E - F R C H D G - - K C I S R Q F V C D S D R D C L D G S D E A S C
123-161 P V L T - C - G P A S - F Q C N S S - - T C I P Q L W A C D N D P D C E D G S D E W Q G
172-210 D S S P - C - S A F E - F H C L S G - - E C I H S S W R C D G G P D C K D K S D E E N C
211-249 A V A T - C - R P D E - F Q C S D G - - N C I H G S R Q C D R E Y D C K D M S D E V G C
250-290 V N V T L C E G P N K - F K C H S G - - E C I T L D K V C N M A R D C R D W S D E P E C
293-324 G T N E - C - L D N N - G G C S - - - H V C - - N D L K I G Y E C L C P D G - - - -
Comp C9 78-115 D D - C - - G N D - F Q C S T G - - R C I K M R L R C N C D N D C G D F S D E D D C
Factor 1 201-238 Q K A D S P - - M D D F F Q C V N G - - K Y I S M K A - C D G I N D C G D Q S D E L - C
239-275 - - C K A C - Q G K G - F H C K S G - - V C I P S Q Y Q C N G E V D C I T G E D E V G C
Unc-52 145-183 Q A G Q - C - M A D E - K A C G N N - - E C V K N D Y V C D G E P D C R D R S D E A N C
186-224 I S R - - C - E P N E - F K C N N N - - K C V Q K M W L C D G D D D C G D N S D E L N C
229-268 S S S D - C - K P T E - F Q C H D R - R Q C V P S S F H C D G T N D C H D G S D E V G C
Perlecan 195-236 F P R V - C - T E T E - F A C H S Y - N E C V A L E Y R C D R R P D C R D M S D E L N C
281-319 G P S A - C - G P Q E - A S C H S G - - H C I P R D Y L C D G Q E D C R D G S D E L G C
321-359 S P P P - C - E P N E - F A C E N G - - H C A L K L W R C D G D F D C E D R T D E A N C
364-403 P G E V - C - G P T H - F Q C V S T - N R C I P A S F H C D E E S D C P D R S D E F G C
Consensus Sequence C F C G C I Φ C D G D C D S D E C

Figure 7

Alignment of the C-type lectin domain of the DGCR2 protein with the corresponding domain of 3 other proteins (position in the sequence of these proteins is shown on the left). Amino acids corresponding to the consensus sequence for C-type lectin as described in (48) are boxed. Homologous residues, which are not part of the consensus sequence are in shaded boxes. Amino acids are in the single letter code. Residues which are conserved in character are designated: Φ, aromatic; θ: aliphatic; Ω: either aromatic or aliphatic; O: oxygen-containing. Hu-CD72: Human B cell differentiation antigen (34); Rat-LECH: rat asialoglycoprotein receptor-1 (31); PGCA: human (33) and rat (32) cartilage-specific proteoglycan core protein precursor.


BF1-2EN 91-160
Hu-CD72 232-255
Rat-LECH 152-175
HPGCA 2204-222?
RPGCA
C-Lectin consensus
BF1-2EN 161-217
Hu-CD72 256-280
Rat-LECH 176-200
HPGCA 2229-2252
RPGCA
C-Lectin consensus

5.3 Appendix 3

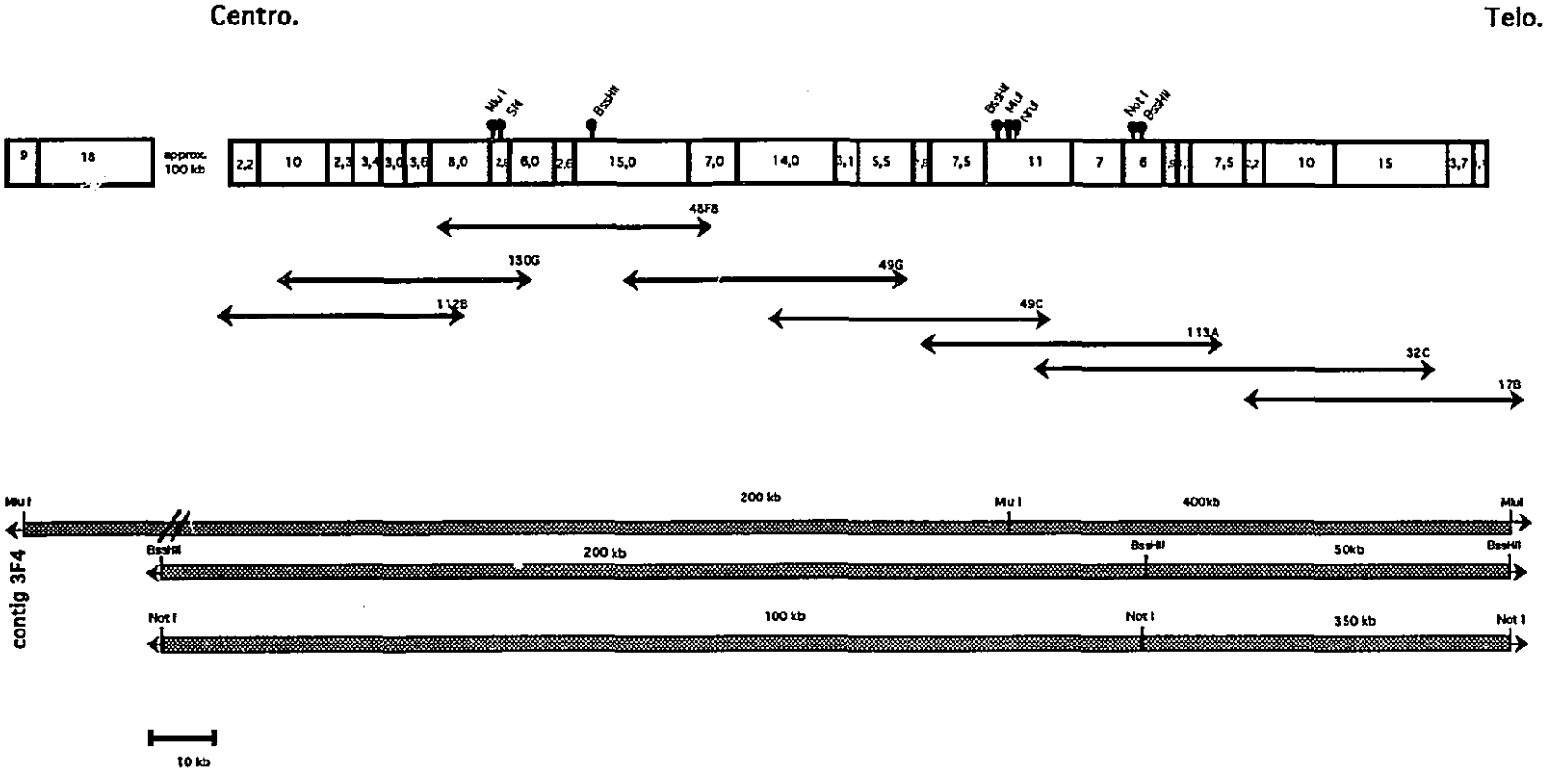
48F8 contig and pulsed-field gel electrophoresis map

The accompanying figure depicts the 48F8 contig, the first cosmid contig constructed in the DGS critical region. The starting cosmid was 48F8 and the contig spans 170 kb of genomic DNA. As the contig was expanded, the position of rare restriction sites was identified, and a pulsed-field gel electrophoresis map was built (shaded bars).

The figure represents the EcoRI restriction map of the region, and each double-headed arrow represents an individual cosmid. The position of the 3F4 contig is indicated, and lies, at the most, 100 kb toward the centromere.

The HIRA gene (Lamour et al., submitted) maps within this contig, from cosmid 49C to 48F8. This gene is completely analogous to TUPLE 1 (Halford et al., 1993a), except that it has 207 extra amino acids in its middle part. It is believed that the TUPLE1 cDNA is a truncated version of the HIRA cDNA, because the size of both the HIRA cDNA and transcript on Northern blot match. RT-PCR products confirmed the size expected from the HIRA gene, but not the TUPLE 1 gene (Lamour et al., submitted). It can thus be concluded that the HIRA protein is the major product transcribed from this locus. HIRA present significant matches with 2 yeast genes (HIR1 and HIR2) which function as repressor of histone transcription.

48F8 CONTIG


Centro.
Telo.
Mu I
Sfi I
BssHII
BssHII
Mlu I
Nru I
Not I
BssHII
approx. 100 kb
9
18
2,2
10
2,3
3,4
3,0
3,6
8,0
2,5
6,0
2,6
15,0
7,0
14,0
3,1
5,5
7,5
11
7
6
7,5
10
15
3,7
48F8
130G
49G
112B
49C
113A
32C
17B
Mu I
200 kb
Mu I
400kb
Mul
BssHII
200 kb
BssHII
50kb
BssHII
contig 3F4
Not I
100 kb
Not I
350 kb
Not I
10 kb

5.4 Appendix 4

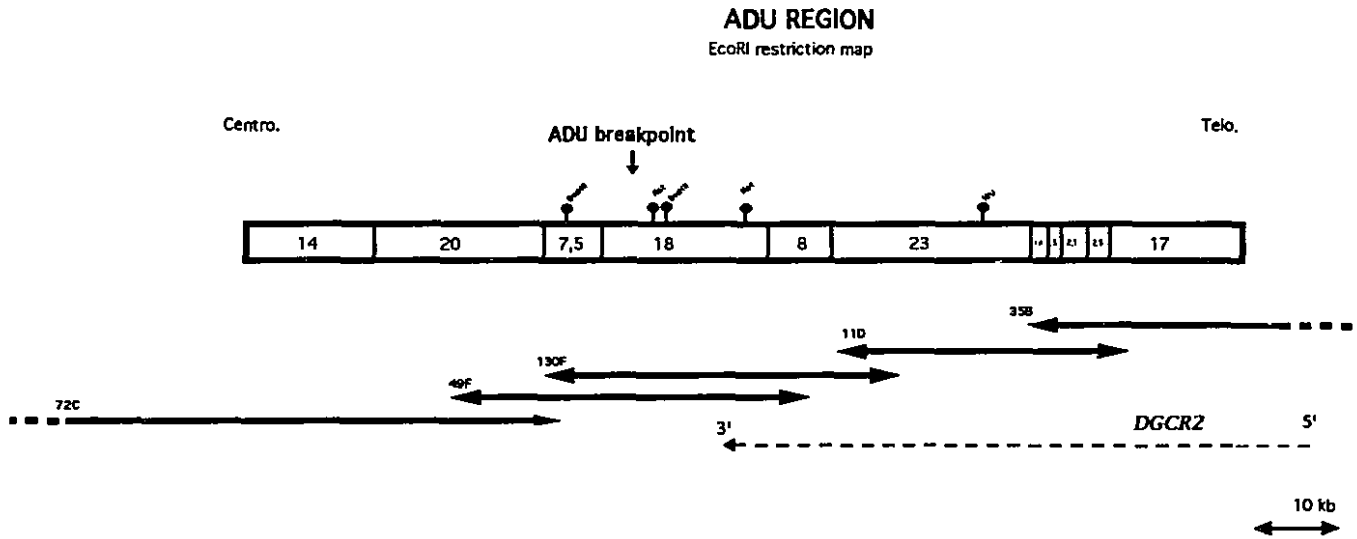
Detailed restriction map around the ADU breakpoint and search for phylogenetically conserved sequences

Once the ADU balanced translocation breakpoint was crossed by chromosome walking, a detailed restriction map of the region was made. Phylogenetically conserved sequences were searched for by sequential hybridization of cosmid fragments to "zoo blots" prepared with human, mouse and rat genomic DNA. The possibility that particular cosmid subfragments map to multiple loci on chromosome 22, as is often the case in this genomic region, was also explored by hybridization to a chromosome 22 hybrid panel. The accompanying figures present these results.

Panel a depicts an EcoRI map of the genomic region around the ADU breakpoint with the position of the overlapping cosmids covering this region and of the DGCR2 gene. Panel b represents a more detailed restriction map of the 14kb, 20 kb and 7.5 kb EcoRI bands lying centromeric to the breakpoint. Panel c represents the detailed restriction map of the 18 kb EcoRI band where the breakpoint lies.

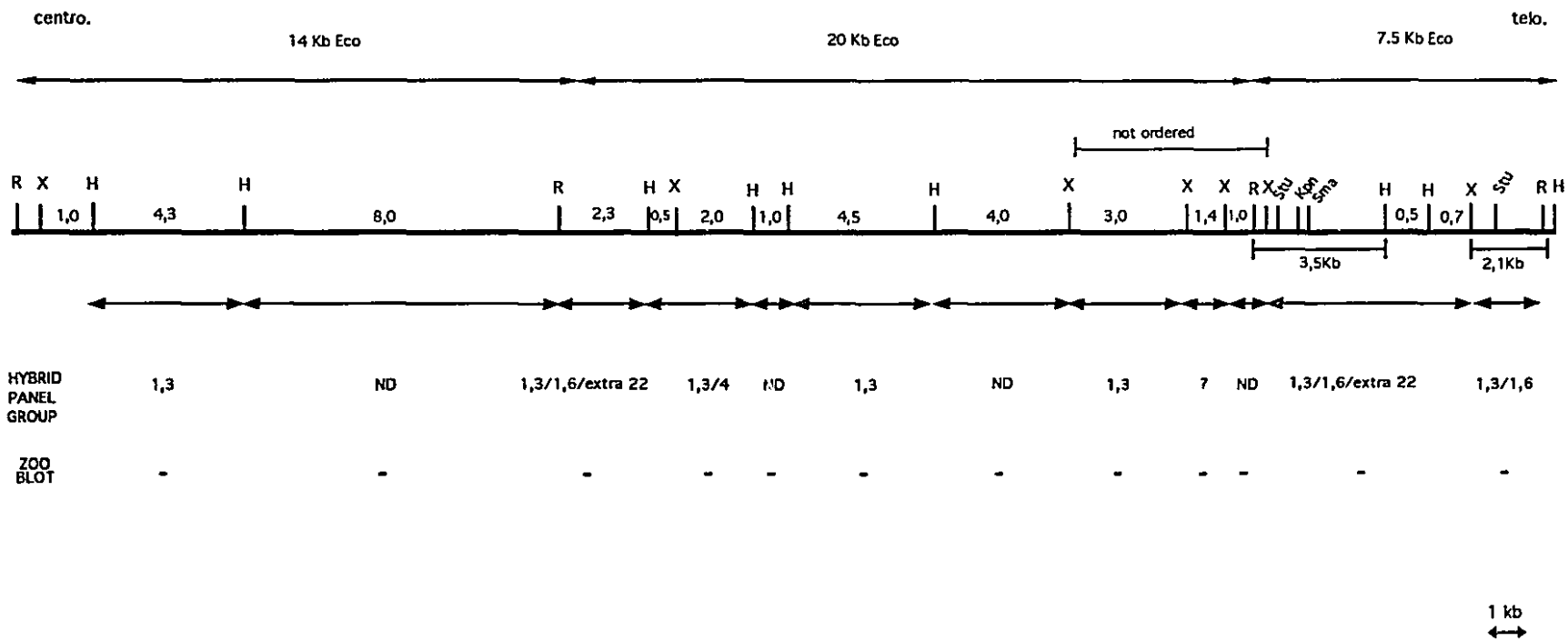
Each EcoRI (R) band was subdivided with other restriction enzymes (X: Xba I, H: Hind III, St: Stu I, Sm: Sma I, Xh: Xho I, K: Kpn I, B: BamHI, N: Not I, Bs: BssHII). Below each subfragments, hybridization results on the hybrid panel and on "zoo blots" are written. Hybrid panel results are written as the group(s) to which a cosmid fragment mapped to. Plus or minus signs for the "zoo blots" results indicate presence or absence of a cross-hybridizing band on rodent DNA.

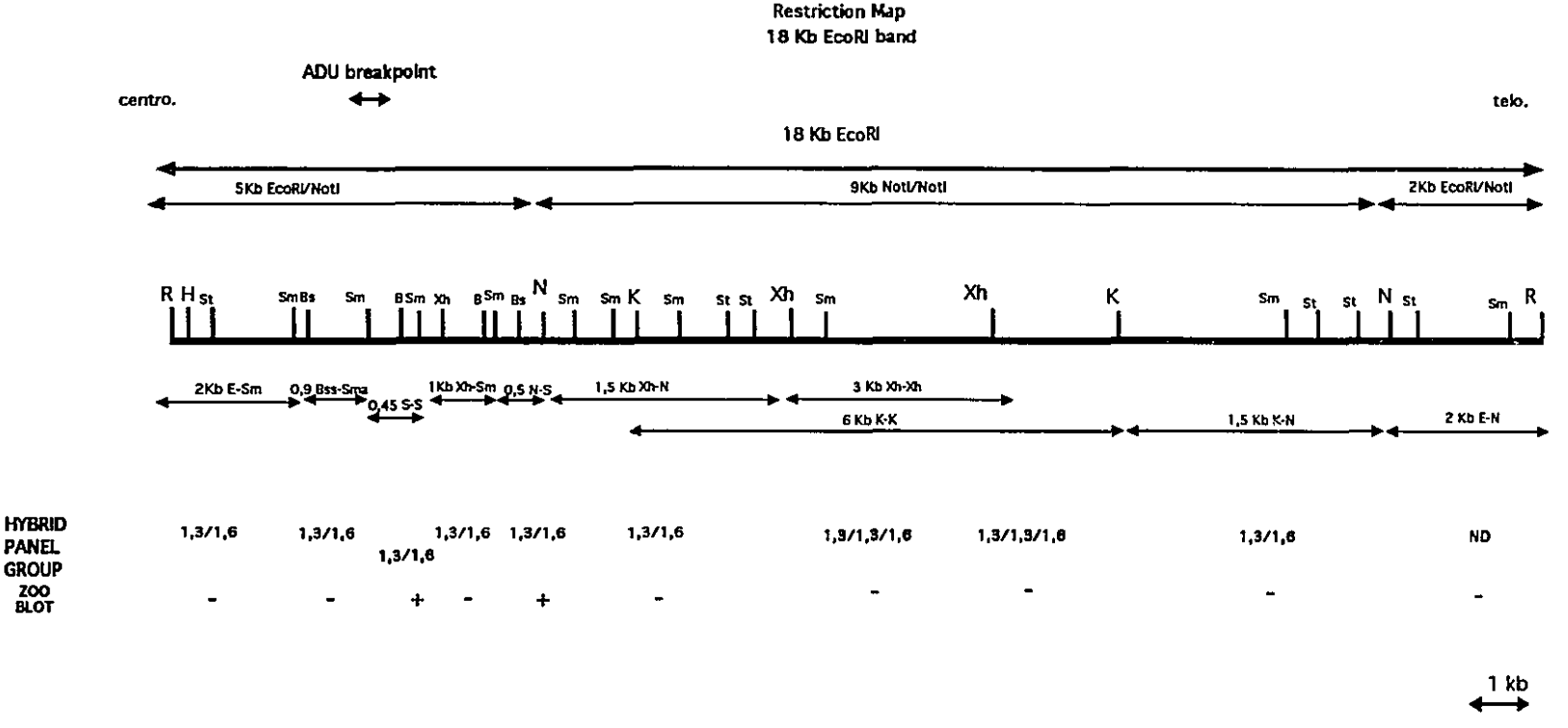
a)


ADU REGION
EcoRI restriction map
Centro.
ADU breakpoint
Telo.
14
20
7,5
18
8
23
17
358
110
130F
49F
72C
3'
DGCR2
5'
10 kb

b)

Restriction map
14 kb, 20 kb, 7.5 kb EcoRI map




Restriction Map
18 Kb EcoRI band
ADU breakpoint
centro.
telo.
18 Kb EcoRI
5Kb EcoRI/NotI
9Kb NotI/NotI
2Kb EcoRI/NotI
R H St Sm Bs Sm B Sm Xh B Sm Bs N Sm Sm K Sm St St Xh Sm Xh K Sm St St N St Sm R
2Kb E-Sm
0,9 Bss-Sma
0,45 S-S
1Kb Xh-Sm
0,5 N-S
1,5 Kb Xh-N
3 Kb Xh-Xh
6 Kb K-K
1,5 Kb K-N
2 Kb E-N
HYBRID PANEL GROUP
1,3/1,6
1,3/1,6
1,3/1,6
1,3/1,6
1,3/1,6
1,3/1,6
1,3/1,3/1,6
1,3/1,3/1,6
1,3/1,6
ND
ZOO BLOT
-
-
+
-
+
-
-
-
-
-
1 kb

CHAPTER 6

6 General Discussion

6.1 Extent of the deletion and phenotypic correlation

This research project has permitted me to characterize genetically a group of developmental syndromes associated with 22q11.2 deletions. The molecular probes used from this chromosomal region detected deletions in a great majority of DGS cases. The FISH technique appears to be the method of choice to uncover the presence of these deletions, since only a blood sample from the patient is needed and the absence of a fluorescent signal on one of the chromosome 22 is easily detected. In addition, FISH can also supplement karyotypic analysis because the presence of an unbalanced translocation can be detected by scoring the number of small acrocentric chromosomes.

Molecular deletions in chromosomal band 22q11.2 were detected in 22 out of 23 (96%) patients studied. No obvious correlation could be drawn between the severity or the number of structures affected, and the extent of the deletion, although it can not be excluded that a more subtle manifestation in the phenotype might correlate with the extent of the deletion. Furthermore, all patients that have normal chromosome complements and that have a molecular deletion of chromosome 22, except one, have the same size deletion, given the resolution afforded by the markers used. The deleted chromosomal region extends from, and includes, Sc11.1a to cos40 (appendix 2). The one patient that has a different extent of deletion, has lost the interval between and including the 2 Sc11.1 loci, but not the cos40 locus.

Twenty-four new sporadic DGS cases have now been studied in the laboratory with the same set of probes. In addition, 11 members of 5 families segregating a chromosome 22 deletion associated with DGS or VCFS phenotypes have beeen recruited in the laboratory (Desmaze et al., 1993a and unpublished data). Except for 3 additional patients that display no chromosome 22 deletions with the probes tested to date, all have lost the genomic region between and including the 2 Sc11.1 loci (Chapter 1, fig. 1). Furthermore, 9 out of 9

patients tested have also lost the cos 40 locus, which maps distal to the constitutional t(11;22) (q23.1;q11.2) translocation breakpoint. Therefore, this result extends the DGS commonly deleted region telomeric to this landmark on chromosome 22.

Other authors did not report consistent deletion of probes distal to the t(11;22) breakpoint in DGS. However, it is likely that in one research group, probes telomeric to the constitutional t(11;22) were seldom studied. In one of the first papers on chromosome 22 deletion screening by this group (Scambler et al., 1991), 3 out of 4 patients tested were found deleted for KI-197, a probe which recognizes a locus mapping telomeric to the t(11;22) translocation breakpoint (Desmaze, unpublished data). In subsequent studies, probes localized telomeric to the Sc11.1b (distal) locus were simply not tested. As for the second research group, a probe mapping telomeric to the constitutional t(11;22) breakpoint (BCRL2) was found deleted in 7 out of 10 patients (Driscoll et al., 1992a). Since the probe used recognizes several loci on Southern blots of genomic DNA, the identification of a band of decreased intensity can be rendered rather complicated (Budarf et al., 1988). In addition, the frequency of deletion for this probe was not assessed in subsequent studies.

The observation that there exists few differences in the extent of the deletions in the different DGS cases analyzed, coupled to the finding of clusters of moderately repetitive sequences flanking the commonly deleted region makes very attractive the hypothesis that the latter are involved in the generation of the DGS deletion. The situation in DGS is reminiscent of that found in Prader-Willi and Angelman syndromes. The majority of patients with either of these syndromes have large deletions in 15q11-q13, with a few rare patients in which the deletion breakpoints are unique. In addition, the proximal and distal deletion breakpoints in these syndromes cluster within the same YACs at either end of the 15q11-q13 region, and probes recognizing multiple loci mapping within the critical region and distal to it have been identified (reviewed in Nicholls, 1993). These repeat families have been proposed to be involved in the occurrence of the 15q11-q13 deletion by a recombinational

event, although no direct proof of this hypothesis are available (Amos-Landgraf et al., 1994).

The implication of the low copy repeat families in the generation of the DGS deletion through a non-homologous recombination event would be interesting to investigate in the future. Cloning of the deletion borders in a DGS patient and comparison of the sequence at this breakpoint with deletion breakpoints of other DGS patients is an area of current investigation in the laboratory.

6.2 Parental origin of the deletions

The finding of a large excess of deletions of maternal origin in the CATCH 22 group of syndromes is intriguing. The study of the inheritance pattern of a microsatellite in sporadic cases of DGS and subsequent pooling of data from the literature has demonstrated definitively that the parental origin of the 22q11.2 deletions is nonrandom (70 deletions of maternal origin against 21 of paternal origin). Between the first submission of this paper and receipt of the reviewer's comments, 11 new cases of sporadic or familial CATCH 22 have been published, all with deletions transmitted by the maternal parent, further supporting our finding.

Nevertheless, there are limits to this analysis. We have pooled in the X^2 tests performed, sporadic, familial and unbalanced translocation cases of CATCH 22. Furthermore, we have computed parental origin of a phenotype, in cases published before the advent of molecular diagnosis, i.e. on the basis of clinical suspicion in one of the parents, without molecular confirmation of a deletion. It is possible that the sample size is too small in each of the categories studied (sporadic: 32 cases; familial with molecular deletion confirmed: 33 cases; familial with clinical suspicion for one parent: 18 cases; inherited unbalanced translocations: 8 cases). It might not be legitimate to pool together sporadic, familial and translocation CATCH 22, because they might correspond to different mechanisms. In addition, we have pooled together all the different phenotypes

designated by the CATCH 22 acronym, because again, sample sizes in each phenotypic categories are small (DGS: 52 cases; VCFS: 30 cases; isolated conotruncal cardiopathies: 9 cases). However, some difficulties are encountered when trying to categorize each of the cases: either these cases were reported as DGS, before VCFS was deemed to exist, or the DGS and the VCFS phenotypes both segregate within the same families.

Different hypotheses can be invoked to explain this preferential maternal origin of the deletions. Affected males may have a lesser reproductive success or decreased fertility compared to females. To test this hypothesis, one would have to determine the parental origin of the deletion in the grandparents of the proband, when one of the parents have a deletion.

An alternative hypothesis would be that this genomic region is subjected to imprinting, i.e. that there is differential expression of the genes depending on their parental origin. The situation in DGS would then be reminiscent of Angelman syndrome, where 15q11-q13 deletions are exclusively found on the chromosome of maternal origin. However, the putative imprinting mechanism in DGS would be rather "leaky", since 23% of DGS deletions are of paternal origin. A potential mechanism by which the "imprinting hypothesis" would work in DGS is that the paternal allele would have a generally reduced activity compared to the maternal one, and influences of the genetic background of the individual would account for DGS arising through paternal deletions.

Imprinting might modulate the phenotype, i.e. isolated conotruncal cardiopathy versus VCFS vs DGS. The results from the data outlined in Appendix 1 of chapter 3, where the VCFS appears to have a slightly greater tendency (albeit not significant) than DGS to be caused by deletions of maternal origin (VCFS: 26 maternal cases, 4 paternal cases; DGS: 33 maternal cases, 11 paternal cases) suggests a trend. Nevertheless, as mentioned previously, it is sometimes difficult to assign a patient to a particular phenotypic category. A more refined measure of the degree of severity of the phenotype could be calculated by devising a score for each of the features of

CATCH 22. This calculated index could then be correlated with the parental origin of the deletion.

In the event of an imprinting mechanism acting on the expression of the genes in the DGS region, the paternal origin of the deletions could be associated with a very severe phenotype, incompatible with survival, or alternatively, with a phenotype so mild that these CATCH 22 cases go unnoticed. Alternatively, the paternal origin of the deletions could be explained by the existence of allelic variation at loci responsible for imprinting. Such "imprinting mutations" in cis- or trans-acting loci, resulting in a failure to reset the imprint in the parental germ line have been reported before for known imprinted loci in the human genome [Weksberg et al., 1993, (Insulin-like growth factor 2 in Beckwith-Wiedemann syndrome); Reis et al., 1994, (Prader-Willi and Angelman syndromes); Forejt and Gregorova, 1992, (mouse t-locus)]. Such an aberrant imprinting of the maternal allele that renders it inactive or less functionnal could thus explain why the CATCH 22 phenotype can occur with paternal deletions.

However, no direct evidence to date indicates that chromosome 22 contains any imprinted genes. In a recent paper, Schinzel et al. (1994) reported 3 cases of maternal uniparental disomy for chromosome 22 with normal phenotype, demonstrating that no maternally imprinted genes with deleterious effect map to chromosome 22. Nonetheless, these results do not exclude the possibility of paternally imprinted genes on chromosome 22. The fact that the deletion is predominantly of maternal origin in DGS/VCFS suggests that the maternal allele is active (or the most active), a hypothesis consistent with the data on chromosome 22 maternal uniparental disomy.

The imprinting of loci has been demonstrated to be conserved through evolution. Many loci on the proximal part of chromosome 22 have been mapped to mouse chromosome 16, notably 3 genes from the DGS/VCFS commonly deleted region, and one gene distal to it (Bucan et al., 1993; Halford et al. 1993b; Mattei et al., 1994). However, normal complementation seems to prevail for the whole length of mouse chromosome 16, as shown by the recovery in the

expected frequencies of mice disomic either for the maternal or the paternal chromosome 16 (Berger and Epstein, 1989). Nevertheless, these data are based only on frequencies analysis at birth and do not include morphological data. Thus, they do not represent a definitive argument against the presence of imprinted genes on mouse chromosome 16.

It would be interesting to examine further the possibility that the 22q11.2 genomic region is subjected to imprinting. This hypothesis can be investigated using three approaches. The first one would be to look for replication asynchrony of this genomic region, since all known imprinted genes in humans and mice have one of the parental allele usually replicating before the other one (Kitsberg et al., 1993). Since these imprinted genes seems to be embedded in 1-2 Mb chromosomal regions showing differential DNA replication, it is possible to measure replication timing using fluorescence in situ hybridization of probes mapping in this region, on unsynchronized interphase nuclei by counting the number of nuclei showing two single spots, two doublets and a single-double hybridization pattern. The imprinting of genes in the region could also be explored by looking for epigenetic differences in DNA methylation between the 2 parental alleles using methylation sensitive restriction enzymes (Nicholls, 1994). Finally, a search for polymorphisms in 3' and 5' untranslated regions of genes in the region could be undertaken to examine the possibility that these genes are expressed from only one parental allele (functional imprint) (Nicholls, 1994).

Finally, the higher frequency of deletions of maternal origin could be explained by some particular structural features of the chromatin allowing the maternal chromosome to be more liable to delete, for example an excess of maternal non-homologous recombination. A similar mechanism has been proposed to explain the fact that only paternal de novo duplications, containing both paternal haplotypes, are observed in sporadic Charcot-Marie-Tooth disease type 1A patients (Palau et al., 1993). Furthermore, an indirect evidence for a parent of origin bias involving human chromosome 22 has come from the observation of an unexplained preferential rearrangement of the chromosome 22 in the generation of the BCR-

ABL fusion gene associated with chronic myeloid leukaemia. When cytogenetic heteromorphism of chromosome 22 are examined by silver staining of the nucleolus organizing regions (Ag-NOR), the maternal BCR gene always seems to be involved in the specific translocation with ABL (11 cases), whereas when a RFLP in the BCR is used, the rearranged allele is always paternal (3 cases) (Haas et al., 1992; Litz and Copenhaver, 1994). Furthermore, there appears to exist a different chromatin configuration around the abnormal BCR/ABL fusion gene: the pattern of DNAase I hypersensitivity is modified when compared to the normal BCR allele (Schaefer-Rego et al., 1990). This was suggested to result in an inappropriate regulation of the BCR/ABL gene expression. Nevertheless, there is no evidence of any functional imprinting of the two parental BCR alleles (Riggins et al., 1994).

6.3 Physical mapping of chromosomal band 22q11.2

The mapping of microsatellite markers on chromosome 22 was undertaken to increase the probe content of the DGS deleted region. These probes could also have been useful for a PCR-based diagnosis of CATCH 22. If many CA-repeats would have been found to map within this region, these could have been used for diagnosis, in cases where only a small quantity of frozen blood from a patient is available. Homozygosity for 5 CA-repeats each having a frequency of heterozygosity greater than 70%, gives a likelihood that the patient harbours a deletion in greater than 99% of cases. A PCR-based screening could be useful to determine the frequency of microdeletions in population of patients with only few characteristics of the CATCH 22 syndrome (e.g. sporadic isolated conotruncal cardiopathies or immunodeficiencies or hypoparathyroidism or facial dysmorphism,...). Nevertheless, this method would not provide any indication of the presence of a chromosome rearrangement, and should be followed by FISH and karyotype analysis in cases where a deletion is found.

Unfortunately, none of the 37 CA-repeats obtained from the Genethon (France) has been found to map within the DGS deleted region. A search for polymorphic microsatellites in the DGS cosmid contigs constructed in the laboratory is underway. These polymorphic markers could also be used to identify the parental origin of the chromosome 22 deletion in the families that were not informative for the D22S264 marker and to search for uniparental disomy (i.e. inheritance of 2 copies of part or all of chromosomes 22 from only one parent) in cases of CATCH 22 that display no deletions.

Three approaches have been used to build a detailed physical map of the DGS deleted and critical regions. The coupling of these approaches has proven rapid and efficient. First, probes were localized to the proximal part of chromosome 22 using a panel of somatic cell hybrids that divides the DGS deleted region in 4 subgroups.

Next, we took advantage of the existence of DGS patients bearing chromosomal translocations to map cosmid loci relative to their translocation breakpoints. Because no somatic hybrids were available, metaphase spreads from these patients were hybridized with biotinylated cosmid probes from the region. This approach has proven invaluable to understand the organization of the low-copy repeat families mapping in the region. Very often, the hybridization patterns generated by such probes were very difficult to interpret on Southern blots, because they would recognize bands of the same size, but mapping in different regions of chromosome 22. FISH on metaphase spreads of translocation-bearing DGS allowed us to readily identify their localization.

Finally, two-color FISH was used to order probes mapping within the same subregion, for distance estimation and for cosmid contig orientation. This method allowed us to utilize whole cosmids as probes, instead of having to go through the isolation of single-copy probes needed for the same type of analysis by pulsed-field gel electrophoresis.

The result of this strategy has enabled the refinement of the physical map of the DGS critical region and the fine-mapping of the ADU balanced translocation breakpoint within a 100-300 kb region, a

distance amenable to chromosome walking (Trask et al., 1989). A first cosmid contig was constructed starting from cosmid 48F8, which maps telomeric to the ADU balanced translocation breakpoint (appendix 3). The 48F8 contig spans 170 kb of genomic DNA, but could not be prolonged further than 30 kb in one direction, although the genomic cosmid libraries used represent 8 genome-equivalents. The under-representation of genomic sequences from this region has already been reported (Halford et al., 1993a). Nevertheless, part of the contig has been made available to Dr. M. Lipinski (Institut Gustave-Roussy, France), for the study of the genomic organization of the HIRA gene, the gene homologous to TUPLE 1 which has been isolated by Halford et al. (1993a).

A second cosmid contig was constructed from the two extremities of the 3F4 cosmid, which maps centromeric to 48F8, but still telomeric to the ADU breakpoint. One side of the contig could not be extended further than 70 kb from the starting cosmid. This turned out to be the telomeric extremity of the contig which lied toward the extremity of the 48F8 contig that also could not be prolonged. Probes from the extremities of both of these contigs hybridized to the same 200 kb MluI band on pulsed-field gel electrophoresis blots prepared with control DNA. The gap between the 2 contigs is, at the most, 100 kb long.

The 3F4 contig covers 325 kb of genomic DNA on chromosome 22 and spans the balanced t(2;22) translocation breakpoint. A detailed restriction map of the region around the breakpoint was constructed and confirmation of breakpoint crossing was obtained from Southern hybridization with small cosmid fragments mapping on either side of the breakpoint, on DNA from the DGS patient and her mother. Bands corresponding to the 2 translocation derivatives were observed on Southern blots, and the rearrangement appears to be balanced. A systematic search for phylogenetically conserved sequences on either side of the breakpoint was performed. A 0.8 kb StuI-StuI fragment from cosmid 130F hybridizes to mouse and rat genomic DNA and was used to isolate cDNA clones from a human fetal brain library. Isolation and characterization of these cDNA clones will be presented below.

Two small fragments from cosmid 49F also hybridized with rat and mouse genomic DNA (appendix 4). These 2 fragments were positioned within 2 kb telomeric from the balanced translocation breakpoint, and very close to a putative CpG island. However, attempts to isolate cDNA clones from a variety of libraries with either of these 2 probes have proven unsuccessful. A subfragment containing these 2 probes (5 kb EcoRI-NotI, cosmid 49F) was subcloned into the pMHC3 exon trapping vector, in both orientations, and the constructs transfected into Cos7 cells (Hamaguchi et al., 1992). After RNA extraction and PCR amplification from both arms of the vector, a product of greater size than the "trapping cassette" alone was observed. Sequencing of the PCR product revealed that a putative exon had effectively been spliced using a splice acceptor site in the insert 38 bp from the EcoRI cloning site, and a cryptic splice donor site within the intron of the trapping cassette. Further experiments involving the cloning of an insert which would have this putative exon in its middle, into the exon trapping vector are pending. Therefore, it can not be excluded with certainty that there is a gene that is effectively interrupted by the balanced translocation breakpoint.

In a collaborative research project with Dr. J. Rommens (Hosp. for Sick Children, Toronto, Canada), we have send DNA from cosmids 11D, 130F, 49F, 72C and 28D (figure 1, chapter 5) to be used for hybridization to cDNA pools by a direct selection method (Rommens et al., 1994). The cDNA libraries used included human fetal liver, kidney and brain and human placenta. Four cDNA clones that appropriately map within the cosmid contig have been retrieved, corresponding to 2 different clone families. Analysis of these clones to determine whether they cross-hybridize to the cDNA already isolated or whether they correspond to new genes mapping in the region is pending.

6.4 Isolation of DGCR2

The characterization of the human fetal brain cDNA clones obtained by screening with the 0.8 kb StuI-StuI conserved probe has lead to the isolation of a novel gene that encodes for a potential transmembrane protein with significant similarities to the Cys-rich repeats of the low-density lipoprotein receptor and to C-type lectin domains. Even though this gene (DGCR2) does not span the balanced t(2;22) translocation breakpoint, there are arguments indicating that it might be a good DGS candidate gene.

First, DGCR2 maps within the DGS critical region and is lost in all DGS patients that bear a chromosome 22 deletion. Hybridization of subfragments of the DGCR2 cDNA and of single-copy probes from the genomic region covering the 3' part of the gene on Southern blots of digested DNA from 3 DGS patients showing no deletions, did not reveal any gross rearrangements. However, more subtle rearrangements or very small deletions cannot be excluded. A more systematic study of these patients using FISH on extended chromatin (DIRVISH), Southern hybridization and reverse transcription PCR is being performed.

DGCR2 maps 10 kb telomeric to the ADU balanced translocation breakpoint and could be directly involved in the pathology of this patient in either of 2 ways. One first hypothesis would be that there exists a position effect resulting from a chromatin structure alteration due to juxtaposition of chromosome 2 material close to DGCR2, and inhibiting its expression. Similar gene "silencing" hypothesis have been proposed for the occurrence of a r(15) and Prader-Willi syndrome (Glenn et al., 1993), or for a balanced translocation associated with an aniridia family (Jordan et al., 1992). In both cases, the breakpoints occur several kilobases away from the putative disease loci, but no direct evidence has been provided to confirm this hypothesis. It is noteworthy that the breakpoint of ADU in 2q14.1 seems to occur very close to the site of telomere fusion of 2 ancestral ape chromosomes that resulted in the formation of human chromosome 2 (Ijdo et al., 1991). Position effect variegation of genes lying adjacent to subtelomeric heterochromatin or to constitutive

heterochromatin in other regions of the genome have been documented in mice, Drosophila and yeast (Gottschling et al., 1990; Aparicio et al., 1991; Capel et al., 1993; Dorer and Henikoff, 1994). In a recent study, the proximity of a 3.2 kb repeat to a subtelomeric sequence has been demonstrated. This 3.2 kb repeat on chromosome 4q, when decreased in number, segregates with the facioscapulohumeral muscular dystrophy (FSHD) in many families. It has therefore been proposed that deletions of the tandem array of the 3.2 kb repeat brings the putative FSHD gene closer to the subtelomeric region of chromosome 4 and may subject it to position effect variegation (Bengtsson et al., 1994; Sun et al., 1994).

An alternative hypothesis for the involvement of DGCR2 in ADU's pathology could be that the breakpoint disrupts the normal regulation of the DGS gene, either by physical separation of, for example, a regulatory element or an imprintor locus from the DGCR2 gene. Both of these situations have been observed in human pathologies; namely, the juxtaposition of an enhancer element from the immunoglobulin heavy chain locus to the *c-myc* gene in the t(8;14) of Burkitt's lymphoma (Hayday et al., 1984), and deletion of a putative imprinting control region giving rise to Prader-Willi syndrome (Sutcliffe et al., 1994).

The predicted DGCR2 protein probably possesses a signal peptide and a transmembrane domain, with an extracellular N terminus, and a short cytoplasmic carboxy terminus. In addition, DGCR2 show significant homologies to the putative ligand-binding domain of the low-density lipoprotein receptor (LDLR) and to calcium-dependent lectin domains. The ligand-binding domain of the LDLR and LDLR related protein (LRP) is composed of a highly conserved regular repeat pattern of 6 cysteine residues separated by 7 amino acids. These cysteine residues form 3 disulfide bonds (Goldstein et al., 1985). The several negatively charged amino acids between the 4th and 6th cysteine residues are important for the high affinity of the receptors to their ligands, in allowing positively charged molecules to bind to the receptor. This type of cysteine-rich repeats has been found in a number of other proteins, with diverse functions, such as host defense, lipid metabolism, basement

membrane assembly and cellular adhesion (Stanley et al., 1986; Catterall et al., 1987; Noonan et al., 1991; Rogalski et al., 1993; Krieger and Herz, 1994).

C-type lectin domains are able to recognize and bind to carbohydrates present on the cell surface in a calcium-dependent manner (Feizi, 1991). This domain is found again in proteins involved in diverse biological functions (intracellular routing of glycoproteins, cell-cell adhesion and phagocytosis) (Drickamer and Taylor, 1993). The range of carbohydrates expressed on cell surface changes throughout differentiation. Some carbohydrates are expressed in such a restricted temporal and spatial manner that they can qualify as stage-specific differentiation markers. C-type lectins, as carbohydrate recognizing domains, could be part of the recognition system for these markers (Feizi, 1991). Adhesion receptors, containing a C-type lectin domain have been shown at least to play a role in adhesive interaction of lymphocytes and vessel wall during recirculation (Stoolman, 1989).

The significant similarities of DGCR2 to C-type lectin domains and to the ligand-binding cysteine-rich repeats of the LDLR allow to speculate on an involvement of this gene in the pathogenesis of DGS. We have seen that DGS probably arises as a result of defective migration of the neural crest cells in the pharyngeal pouches or defective interaction with these structures. The ability of neural crest cells to migrate in certain interstitial spaces is promoted or inhibited by the presence of specific glycoproteins (e.g. laminin, fibronectin, keratan sulfate proteoglycan) (Marusich and Weston, 1991). The interaction between neural crest cells and extracellular matrix after migration has terminated is also important; crest cells must express appropriate surface receptors in order to interact with their changing micro-environment. It is therefore possible that the C-type lectin domain of DGCR2 guides a subpopulation of neural crest cells in the pharyngeal pouches, with the LDLR-homolog cysteine-rich repeat providing the specificity necessary for this recognition process.

It is noteworthy that DGS has been found in association with X-linked Kallmann syndrome (Shen et al., 1979). This disease is characterized by a deficiency in hypothalamic gonadotrophin-

releasing hormone (LH-RH) resulting in hypogonadism and anosmia. The gene for this syndrome has recently been cloned, and shares homology with neural cell adhesion and axonal pathfinding (Franco et al., 1991; Legouis et al., 1991), and it has been suggested that the KAL gene is involved in neuronal migration.

To test the hypothesis that haploinsufficiency for the DGCR2 gene is etiologic in DGS, one would need to inactivate one copy of this gene by homologous recombination in mouse embryonic stem cells, and to look at the phenotype of the resulting mice. It is expected that, as early as the F1 generation, chimeric mice would show the DGS phenotype. Patients for which no deletions have been found in the 22q11.2 region could also be screened for point mutations within the DGCR2 gene.

Claims to originality

This thesis contains the following original results:

1. The finding of a 22q11.2 molecular deletion in 96% of patients studied.

2. The determination that the DiGeorge syndrome commonly deleted region extends telomeric to the t(11;22) recurrent constitutional translocation breakpoint.

3. The detailed physical mapping of the DiGeorge syndrome commonly deleted and critical regions.

4. The finding of a great excess of deletions on the maternally inherited chromosomes in DiGeorge syndrome/Velo-Cardio-Facial syndrome patients

5. The cloning by a chromosome walk of a balanced translocation breakpoint that maps within the DiGeorge syndrome critical region.

6. The isolation of a novel gene, DGCR2, that potentially encodes for an adhesion receptor protein.

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