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Progress in the Molecular Cytogenetics of Man

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Progress in the molecular cytogenetics of man

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[Plate 1]

Recombinant DNA technology has contributed greatly to the precision of chromosome analysis in man. Breakpoints of chromosome deletions and rearrangements may be defined on a chromosome map whose landmarks are the loci of DNA sequences rather than Giemsa bands. Flow cytogenetics allows the extent of chromosome duplications and deletions to be measured more precisely than has hitherto been possible, DNA probes can reveal hidden translocations through the application of *in situ* hybridization, and may be used as markers to determine the parental origin of non-disjunction. It is evident that a study of the pathology of human chromosomes now requires the combined skills of recombinant DNA and cytology.

Introduction

Human cytogenetics came of age in 1956, the year that Tjio and Levan realized that the diploid chromosome number in man was 46 rather than 48. However, the importance of cytogenetics to human genetic abnormalities was not appreciated until Lejeune *et al.* (1959) reported an extra chromosome in Down's syndrome, and this was quickly followed by the discovery of human sex chromosome aberrations and translocation Down's syndrome. With the introduction of the short-term lymphocyte culture technique by Moorhead *et al.* (1960) it became a simple matter for pathology departments all over the World to count chromosomes from patients with major mental and physical handicap, with recurrent miscarriage, with infertility, and with leukaemia and related disorders. Within a few months trisomy 18 (Edwards's syndrome), trisomy 13 (Patau's syndrome), and the Philadelphia chromosome in chronic myeloid leukaemia had been discovered. However, many severely disabled children, with dysmorphic features similar to those found in trisomic cases, were shown to have apparently normal chromosomes and it became clear even in those early days that methods of chromosome identification and analysis were too crude to recognize more than very gross changes in chromosome length. The diagnosis of smaller aberrations could only be expected if there were improvements in technique, and so it has been proved. At first only chromosomes 1, 2, 3, 16 and the Y-chromosome could be unambiguously identified on the basis of length and centromere position (Denver Conference 1960), the others were assigned to the alphabetical groups A to G, a system that persisted for the next 10 years. As familiarity with human chromosomes grew, a few investigators were able to distinguish vague, inconstant features of particular chromosomes, which were referred to as 'secondary constrictions' and which seemed useful for chromosome identification (Ferguson-Smith *et al.* 1962). These features were later found to be manifestations of heterochromatin situated near to the centromeres of chromosomes 1, 9 and 16 and less frequently at other sites on chromosomes 6, 11, 17 and the Y. At this time, others realized that the patterns of DNA replication as revealed by radioautography after

pulse-labelling with tritiated thymidine (German 1962; Schmid 1963) could also be used in chromosome identification. This was a labour-intensive exercise and the demonstration by Caspersson *et al.* (1969) that quinacrine mustard staining could generate chromosome-specific fluorescent bands under ultraviolet light was rightly hailed as a major technical breakthrough. The 'Q' banding method was quickly followed by 'G', 'C' and 'R' banding techniques which depended on a variety of treatments including heat, alkali, trypsin digestion and detergents to produce more permanent specific banding on chromosomes. By 1971, no reputable cytogenetics laboratory could provide a chromosome diagnosis without using at least one of these banding techniques, and the improved resolution afforded by the new methodology resulted in the recognition of many 'partial trisomy' and 'deletion' syndromes. Over 50 such chromosomal syndromes have since been catalogued. The banding techniques have continued to become more sophisticated and the earlier preparations that could resolve about 300 chromosome bands have given way to prometaphase banding techniques that can resolve 800–1000 bands.

Today, chromosome methodology has reached a stage of resolution that allows the occasional identification of deletions and duplications involving about 0.2% of the haploid genome, equivalent to approximately 6×10^6 base pairs (b.p.) (6 megabases (Mb)). The majority of such changes must still pass unrecognized by routine karyotype analysis unless they involve a particularly prominent band, or result in reduced dosage of a gene product whose structural locus has already been mapped to that region. For example, chromosome deletions associated with retinoblastoma have sometimes been recognized by first demonstrating reduced activity of esterase D which maps to the same region. There is therefore a need for more reliable cytogenetic techniques that will detect chromosome aberrations in the range of 1–10 Mb, and also for molecular genetic techniques that help to bridge the resolution gap between 1 b.p. and 1 Mb.

It is conventional to define chromosome aberrations as genetic mutations that can be observed with the light microscope. This serves to emphasize that the only difference between deletions, duplications, inversions and translocations at the molecular and cytogenetic levels is one of scale. The causes of both types of mutation are probably the same, by far the majority occurring as accidents during meiotic recombination and the remainder as a result of mutagenic agents such as radiation. Whatever the causes, the recognition of even smaller aberrations in patients with genetic disease must in future depend on the approaches now being developed in molecular genetics.

Flow cytogenetics

One of the most promising of the new techniques for measuring DNA content in normal and abnormal chromosomes applies the techniques of flow cytometry to suspensions of chromosomes. A fluid suspension of chromosomes stained by a fluorescent dye is passed at a steady rate through the laser beam of a standard fluorescence activated cell sorter (FACS). The laser generates fluorescence in the chromosome which can be detected and collected by photomultipliers and stored in a computer. Up to 2000 chromosomes can be analysed per second and each chromosome produces one pulse at a particular intensity of fluorescence. A histogram (figure 1), or flow karyotype, is produced which shows a series of peaks at increasing levels of fluorescence. The area of each peak corresponds to the relative number of chromosomes

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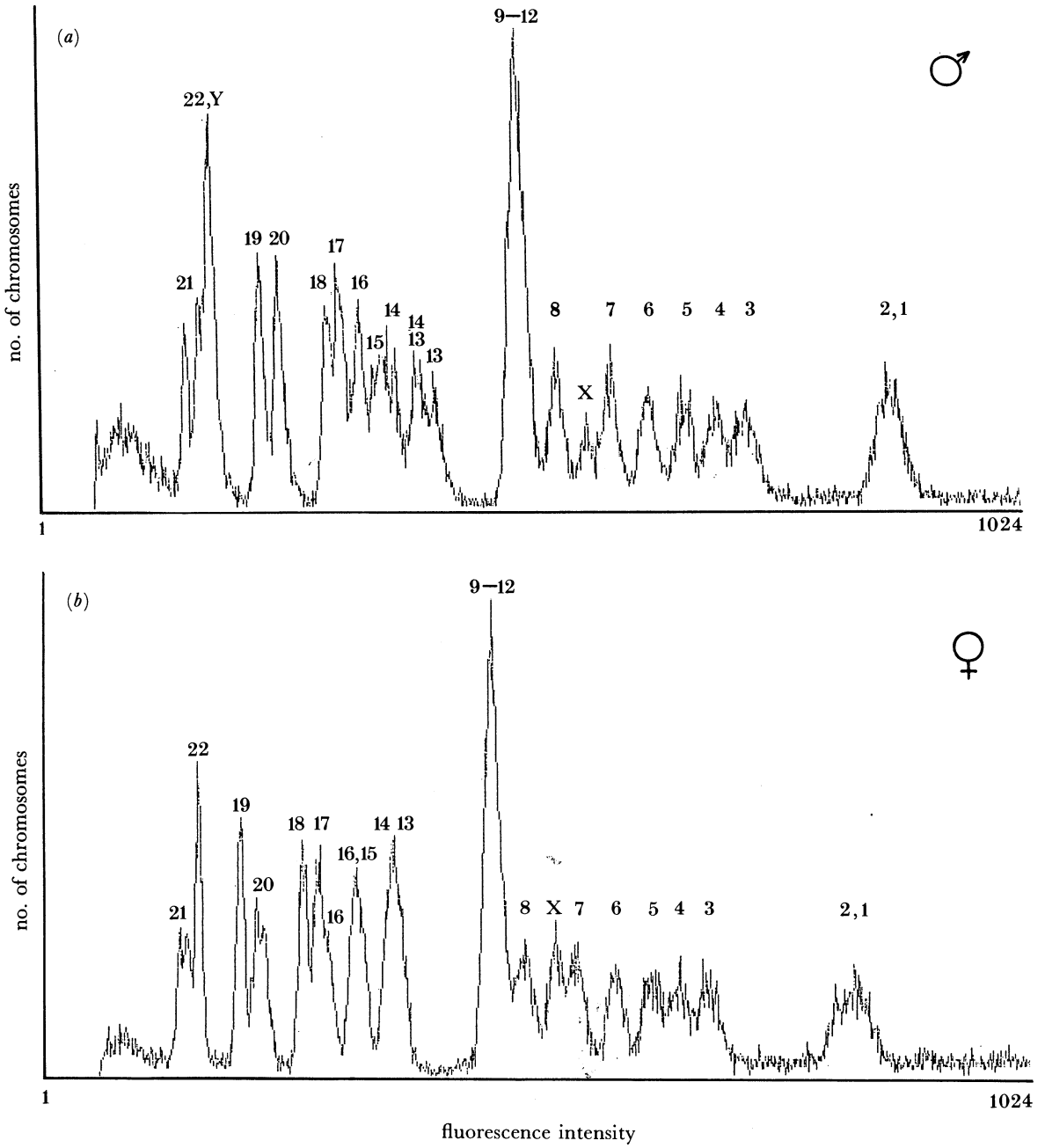

FIGURE 1. Flow karyotypes from normal male (*a*) and female (*b*) cells. Note the difference in area of the X-chromosome peak.

(pulses) which have similar fluorescence, the median of each peak representing the relative fluorescence for that group of chromosomes. If the chromosomes are stained uniformly by the dye (e.g. ethidium bromide) the relative fluorescence of each chromosome group is equivalent to the DNA content. A number of chromosomes, including 3–8 and the X, form individual peaks and it is possible to determine their DNA content rather precisely (Harris *et al.* 1986). Others, notably 1–2 and 9–12, have similar DNA content and usually cannot be resolved

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separately. Many chromosomes show considerable variation in DNA content due to variation in centric heterochromatin and other non-transcribed repetitive DNA and this effects particularly chromosomes 1, 9, 16, the Y-chromosome and the satellited chromosomes 13–15 and 21–22 (Young *et al.* 1981). Distinction between normal variation and a significant chromosome aberration therefore requires studies of families (Cooke *et al.* 1987). Fortunately, the X-chromosome is least variable in DNA content and the measurement of DNA duplication and deletion is comparatively straightforward. The value of flow cytogenetics is illustrated here by its use in measuring the extent of microdeletion of Xp21.2 in cases of X-linked muscular dystrophy and in measuring the extent of X-Y interchange in X-Y interchange males.

Microdeletion of Xp21.2

It has been shown by Southern blotting with a series of DNA probes which map to Xp21.2 that perhaps as many as 7% of boys affected with X-linked muscular dystrophy have a deletion at the Duchenne and Becker muscular dystrophy (DMD–BMD) locus (Kunkel 1986). Sometimes the deletion is much more extensive and includes loci for other X-linked disorders such as chronic granulomatous disease, glycerol kinase deficiency, congenital adrenal hypoplasia and mental retardation. Such occurrences are usefully termed 'microdeletion syndromes'. The most extensive of these microdeletion syndromes can sometimes produce a deletion visible under the microscope as a narrowing of band Xp21.2 (Franke *et al.* 1985; Wilcox *et al.* 1986). We have studied 13 patients with molecularly defined deletions at Xp21.2 (table 1) and a variety of X-linked disorders. Five patients (M.J., S.S., J.D., B.B. and A.M. in table 1) show

Table 1. DNA analysis of 13 males with Xp21.2 microdeletions

	M.J.†	S.S.†	J.D.†	B.B.†	S.B.†	S.J.†	N.J.†	S.M.†	P.E.†	J.B.‡	A.B.†	A.K.†	A.M.
familial (f) sporadic (s)	s	f	f	s	.	.	s	s	f	f	.	.	f
99-6	+	+	+	+	+	+	+	+	+	+	+	+	+
B24	+	+	+	+	+	+	+	+	+	+	+	+	−
C7	+	+	+	+	+	+	+	+	+	+	+	+	−
L1.4	+	+	+	+	+	+	+	+	+	+	+	+	−
GMGX11	−	+	+	+	+	+	+	+	+	+	+	+	+
J-BIR	−	−	−	+	+	+	+	+	+	+	+	+	+
87.30	−	−	−	−	−	+	−	−	−	+	+	+	+
87.15	−	−	−	−	−	−	−	−	−	+	+	+	+
87.8	−	−	−	−	−	−	−	−	−	+	+	+	+
87.1	−	−	−	−	−	−	−	−	−	+	+	+	+
XJ2.3	−	−	−	−	−	−	+	+	+	−	−	−	+
XJ1.2	−	−	−	−	−	−	+	+	+	−	−	−	+
XJ1.1	−	−	−	−	−	−	+	+	+	−	−	−	+
HIP25	−	−	−	−	−	−	+	+	+	−	−	−	+
84	−	−	−	−	+	+	+	+	+	+	+	+	+
754	−	−	−	−	+	+	+	+	+	+	+	+	+
GMGX10	−	−	−	−	+	+	+	+	+	+	+	+	+
GMGX12	+	+	+	+	+	+	+	+	+	+	+	+	+
OTC	+	+	+	+	+	+	+	+	+	+	+	+	+
58.1	+	+	+	+	+	+	+	+	+	+	+	+	+
L1.28	+	+	+	+	+	+	+	+	+	+	+	+	+
adrenal hypoplasia	+	−	−	−	−	−	−	−	−	−	−	−	+
glycerol kinase deficiency	+	−	−	−	−	−	−	−	−	−	−	−	−
muscular dystrophy	+	+	+	+	+	+	+	+	+	+	+	+	−
chronic granulomatous disease	−	−	−	+	−	−	−	−	−	−	−	−	−
mental handicap	+	+	+	+	−	−	−	+	−	−	+	−	+

† DMD. ‡ BMD.

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a detectable narrowing of band Xp21, apparently indistinguishable in each case. However, the clinical findings and the results of analysis with DNA probes reveal striking differences. All are mentally handicapped to a greater or lesser degree: M.J. has deletion of 13 specific DNA sequences associated with muscular dystrophy, glycerol kinase deficiency and adrenal hypoplasia, S.S. and J.D. (who are cousins) have deletion of 12 sequences and are affected only with muscular dystrophy, B.B. has lost 11 sequences and has muscular dystrophy and chronic granulomatous disease, whereas A.M. has lost only 3 sequences and is only affected by congenital adrenal hypoplasia. When the DNA content of the deleted X-chromosomes are measured by flow cytometry (table 2), clear differences in the extent of deletion can be recognized which correlate both with the number of DNA sequences deleted and the extent of the patient's disability. Flow cytogenetics can thus resolve differences that cannot be detected by classical cytogenetic techniques, and DNA analysis can define the limits of a cytogenetic abnormality in a way which is impossible by microscopy alone. Note also that the DNA analysis of these five cases of microdeletion allows the order of the genetic loci for the four disorders to be determined on the X-chromosome. The most distal locus must be that for adrenal hypoplasia, followed by glycerol kinase deficiency, muscular dystrophy and chronic granulomatous disease.

TABLE 2. DNA ANALYSIS OF Xp21 MICRODELETIONS BY USING FLOW CYTOMETRY

patient	relative fluorescence	decrease in X (%)	million base pairs
A.M.	619.7	2.6	4.2
S.S.	615.4	3.8	6.1
B.B.	613.8	4.3	7.0
M.J.	608.8	5.9	9.6
S.B.	638.7	—	—
S.J.	647.8	—	—
A.K.	642.5	—	—
S.M.	640.2	—	—
N.J.	642.3	—	—
K.M.	637.4	—	—

Controls (58) $\bar{X}$ 640.6 ± 4.3.

It should be emphasized that although the relative size of the deletions determined by flow cytometry is likely to be reasonably accurate, the absolute size depends on the assumption that the total haploid DNA content of the human genome is constant and equivalent to 3000 million b.p. More accurate quantification of DNA measurement by flow cytometry must await calibration through duplications and deletions that have either been fully sequenced or have had size estimations based on pulsed-field gradient electrophoresis (van Ommen *et al.* 1986) which can resolve DNA fragments between 10–1000 kilobases.

Analysis of X-chromosome size in X-Y interchange males

It is now appreciated that sex reversal in infertile males with an apparently normal female karyotype (so-called XX males) is due to an abnormal recombination in paternal meiosis which results in transfer of male determinants in the non-pairing part of the short arm of the Y-chromosome (Yp) to the paternal X chromosome (Ferguson-Smith 1966). Thus Y-specific sequences which map to Yp have been detected by Southern blotting in the genomes of X-Y interchange males (Guellaen *et al.* 1984; Page *et al.* 1985; Affara *et al.* 1986*a*, *b*; Muller *et al.*

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1986). However, it is difficult to detect cytological evidence of this interchange (Magenis *et al.* 1982) and we have therefore attempted to measure the two X-chromosomes in X-Y interchange males by flow cytometry (Ferguson-Smith *et al.* 1985). This has revealed that 9 out of 15 patients have one X-chromosome measurably larger than the other. The increase in size of the X in these cases is remarkably constant with a mean of $3.8 \pm 0.1\%$ above normal (table 3). This is equivalent to an additional 6.1 Mb (range 6.0–6.3 Mb) estimated on the assumption that the X-chromosome is 5.4% of the total haploid genome and has 162 Mb of DNA. As the Y-chromosome is on average 1.92% of the total haploid genome it should contain about 57.6 Mb of which 20–25% (or 11.5–14.4 Mb) is in the short arm. It follows that at least half of the short arm of the Y is transferred to the X in these X-Y interchange cases and substantially more if there is reciprocal loss of part of the X-chromosome. The latter seems likely because of the fact that many X-Y interchange males have lost that part of the paternal Xp containing the Xg locus (Ferguson-Smith 1966). These observations are consistent with the hypothesis that an abnormal cross-over outside the X-Y pairing segment results in the transfer of a substantial part of the Y-chromosome including male determinants to the X-chromosome with loss of X-chromosome material.

TABLE 3. RELATIVE DNA CONTENT OF X-CHROMOSOMES MEASURED BY FLOW CYTOMETRY

group A				group B	
patient	normal X	large X	increase (%)	patient	X-chromosome
R.H.	641.8	666.2	3.74	N.E.	634.5
J.M.	636.7	661.6	3.91	H.M.	638.5
T.A.	640.0	663.9	3.73	G.C.	639.4
A.G.	637.3	661.3	3.77	A.N.	635.5
J.T.	636.6	660.6	3.77	R.T.	646.1
G.A.	643.9	669.2	3.93	P.P.	637.2
W.B.	640.4	664.5	3.76		
K.S.	641.2	664.4	3.62		
A.P.	640.7	665.6	3.89		
mean ± s.d.	639.8 ± 2.5	664.1 ± 2.7	3.80 ± 0.1		638.5 ± 4.1

In some presumptive X-Y interchange males both X-chromosomes are indistinguishable in size. These cases tend to have fewer Y-specific sequences and some have no demonstrable Y DNA. It is conceivable that in this class of X-Y interchange male, a smaller region of the Y has been transferred, possibly by an abnormal double recombination in which at least one breakpoint is outside the normal pairing segment.

THE APPLICATION OF RECOMBINANT DNA TECHNOLOGY TO CYTOGENETIC ANALYSIS

The changes in DNA content of the X-chromosome revealed by flow cytometry and described above would have been difficult to interpret without DNA analysis by Southern blotting using X- and Y-chromosome-specific probes. In all these cases, samples of the patients' DNA have been digested with appropriate restriction endonucleases into large numbers of different sized DNA fragments which have been separated by agarose gel electrophoresis and transferred to special membranes for DNA analysis. Cloned radiolabelled DNA sequences of

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known origin (probes) have been hybridized to these membranes carrying the patients' DNA to demonstrate the presence or absence of sequences complementary to the DNA probes. This technique, known as Southern blotting, may also be used to determine the breakpoints in chromosomal rearrangements, and so add to the precision of cytogenetic analysis. A particularly elegant example of the application of this technique has recently been provided by Boyd *et al.* (1986) who have studied a series of X-autosome translocations in female patients with DMD–BMD. About 20 patients are now known in whom a *de novo* reciprocal translocation has occurred between Xp21 and a different autosomal region. It appears that the patients are affected with muscular dystrophy because the break on Xp21 causes a mutation in the DMD–BMD locus. In common with most other such X-autosome translocations, the normal X-chromosome in these patients is preferentially inactivated, and this prevents transcription of the normal allele at the DMD–BMD locus. Cytogenetic analysis is unable to distinguish differences in the site of the breakpoint which occurs in Xp21.2 in most cases. However, it is possible to determine the breakpoint by DNA analyses using man–mouse hybrids which have lost the inactive normal X and have retained one of the derivatives of the X-autosome translocation. Boyd *et al.* (1986) showed that the breakpoints in several cases were in different sites, at least 1000 kilobases apart in two cases. Each breakpoint could be localized between two adjacent cloned DNA sequences, demonstrating that molecular genetic techniques will always be required if the limits of chromosome aberrations are to be defined accurately in terms of the human gene map.

A similar result to the one cited above, could also be achieved (perhaps with less effort) with the fluorescence activated cell sorter. An ingenious technique has been devised (Bernheim *et al.* 1983) which uses FACS to sort large numbers of specific chromosomes directly onto hybridization membranes. The 'dot–blots' so produced can be used to assign an unknown single-copy sequence. This is achieved by hybridizing the cloned radiolabelled sequence to a series of 'dot–blots' containing known fractions of the chromosome complement. X-autosome translocations frequently sort independently of the normal X-chromosome, and so the method could be used to define individual breakpoint in the X by DNA analysis.

In situ hybridization

Many cytogeneticists were surprised to learn in 1970 that radiolabelled DNA probes could be hybridized successfully to fixed and air-dried chromosome preparations on microscope slides. Pardue & Gall (1970) successfully hybridized radiolabelled mouse satellite DNA to mouse chromosomes and showed by radioautography that the radiolabelled probe annealed to the centromeric regions. Similar success was obtained with moderately repetitive sequences such as ribosomal RNA. However, attempts to localize single-copy globin messenger RNA were unsuccessful until recombinant DNA technology was applied. It then became feasible to insert single-copy sequences into appropriate phage or plasmid vectors so that large quantities of probe could be cloned and radiolabelled. Two groups independently showed that cloned single-copy genomic sequences could be hybridized successfully to chromosome preparations (Malcolm *et al.* 1981; Harper & Saunders 1981). This was quickly followed by the first report of the regional mapping of a previously unassigned gene (for κ light-chain immunoglobin) by *in situ* hybridization (Malcolm *et al.* 1982). It is now known that successful localization of single-copy cloned sequences is due to the formation of networks over the complementary sequence on the chromosome; vector DNA hybridizes to itself forming a sufficient nucleus

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of radioactivity over the chromosomal site to be detected by autoradiography. *In situ* hybridization has now become the first approach to assigning cloned DNA sequences to individual chromosomes. It is also a powerful tool for studying the origin of unusual chromosome aberrations, as illustrated in the next section.

The fate of Y sequences in X-Y interchange males

Several groups, including my own, have used *in situ* hybridization to demonstrate that Y-specific sequences may be transferred from the short arm of the Y to the tip of the short arm of the X in X-Y interchange males (Magenis *et al.* 1984; Andersson *et al.* 1986). In the course of isolating and characterizing Y-specific sequences which map to the short arm of the Y (Affara *et al.* 1986*b*), two Y-specific probes, GMGY7 and GMGY10, were isolated which were particularly suitable for *in situ* hybridization as they recognized two distinct Y-specific clustered repeated sequences. It has been shown that these are present in the genomic DNA from 8 of our series of 25 X-Y interchange males. All 8 males have been shown to have a large X-chromosome by flow cytometry, and all can be shown by *in situ* hybridization to have these two sequences on the tip of the short arm of one X-chromosome (figure 2, plate 1). Other sequences are currently being used to confirm X-Y interchange in the remaining patients.

A particularly satisfying experiment using the same probes has been used to prove that a minute chromosome fragment was derived from the Y-chromosome. The patient was an adult male who appeared to have a 45,X karyotype plus a minute fragment that was present in 25% of lymphocytes in a peripheral blood culture. Apart from being extremely short in stature (147 cm), the patient had no other features of Turner's syndrome. When his chromosomes were probed with GMGY7, 7.5% of all silver grains in the radioautographs occurred directly over the minute fragment, incidentally completely covering the fragment in many cells. It seems likely that the testis determing factor (TDF) locus is present with GMGY7 in the tiny fragment and is responsible for the normal male differentiation in this patient. Attempts are being made to separate this fragment from the other chromosomes in order to make a genomic library rich in TDF, the ultimate aim being to clone and characterize the sequences responsible for primary sex determination.

The use of DNA analysis in determining the parental origin of non-disjunction to Down's syndrome

The final example of the application of molecular methods to human cytogenetics relates to the use of DNA polymorphisms (restriction fragment length polymorphisms (RFLPs)) in determining the parental origin of the extra chromosome 21 in Down's syndrome. Several chromosome-21-specific probes have now been isolated that recognize comparatively frequent RFLPs with particular restriction enzymes (Stewart *et al.* 1985). The absence of a particular restriction enzyme cleavage site (or the presence of a 'new' site) can be detected by Southern blotting. An individual may be shown to be heterozygous for chromosome 21 DNA fragments of different size recognized by a particular probe, or be homozygous for either fragment. A Down's syndrome patient may also be heterozygous, but in this case two of the three chromosomes 21 will show the same polymorphism and the third will show the alternative polymorphism. This is revealed in a Southern blot as a difference in dosage, the more intense band on the radioautograph signifying a 'double dose' of the particular chromosome (figure 3). If the parents are homozygous for different RFLPs for which the affected child is heterozygous, it can clearly be seen which parent contributed the double dose and therefore in

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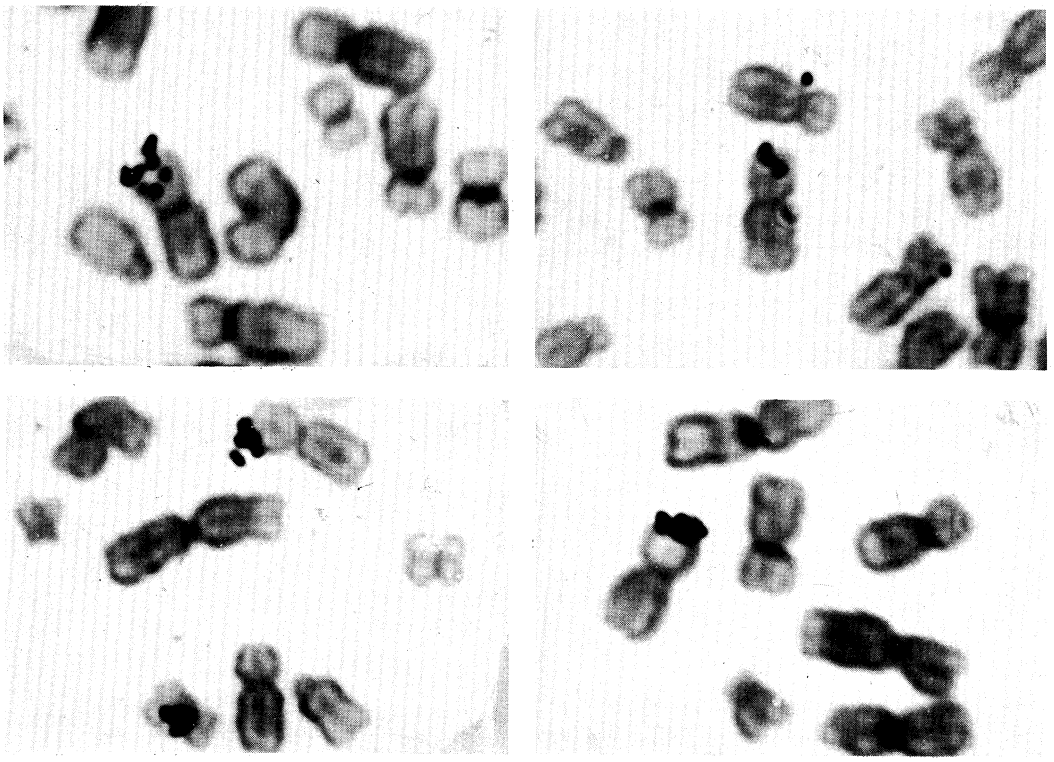

FIGURE 2. *In situ* hybridization of Y-specific probe GMGY10 to the distal end of the short arm of one X-chromosome in an X-Y interchange male.

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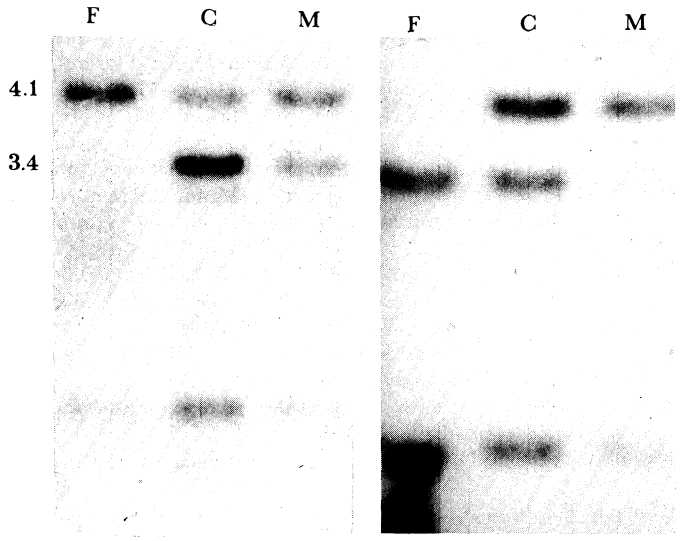

FIGURE 3. Southern analysis of *Msp*I RFLPs recognized by probe D21S15 in two Down's syndrome families. Maternal non-disjunction is indicated in both cases by the increased intensity of the band produced by two maternal chromosomes 21. (F, father; C, Down's syndrome child; M, mother).

whom the non-disjunction event must have occurred (e.g. figure 3). We have so far tested 22 families in this way with a series of 5 different probes, and 9 have been informative. In 7 (78%) the extra 21 is maternal and in 2 it is paternal. This is perhaps not surprising because of the well-known maternal age effect, and the earlier studies using cytogenetic markers that show a similar proportion. It is unfortunately not possible to distinguish meiotic non-disjunction from post-fertilization non-disjunction or to distinguish first meiotic events from second meiotic events (because of crossing-over). The use of a series of chromosome-21-specific probes in these families should, however, answer questions about the role of non-conjunction in the aetiology of Down's syndrome, as such occurrences should reduce the frequency of recombination between linked markers on chromosome 21.

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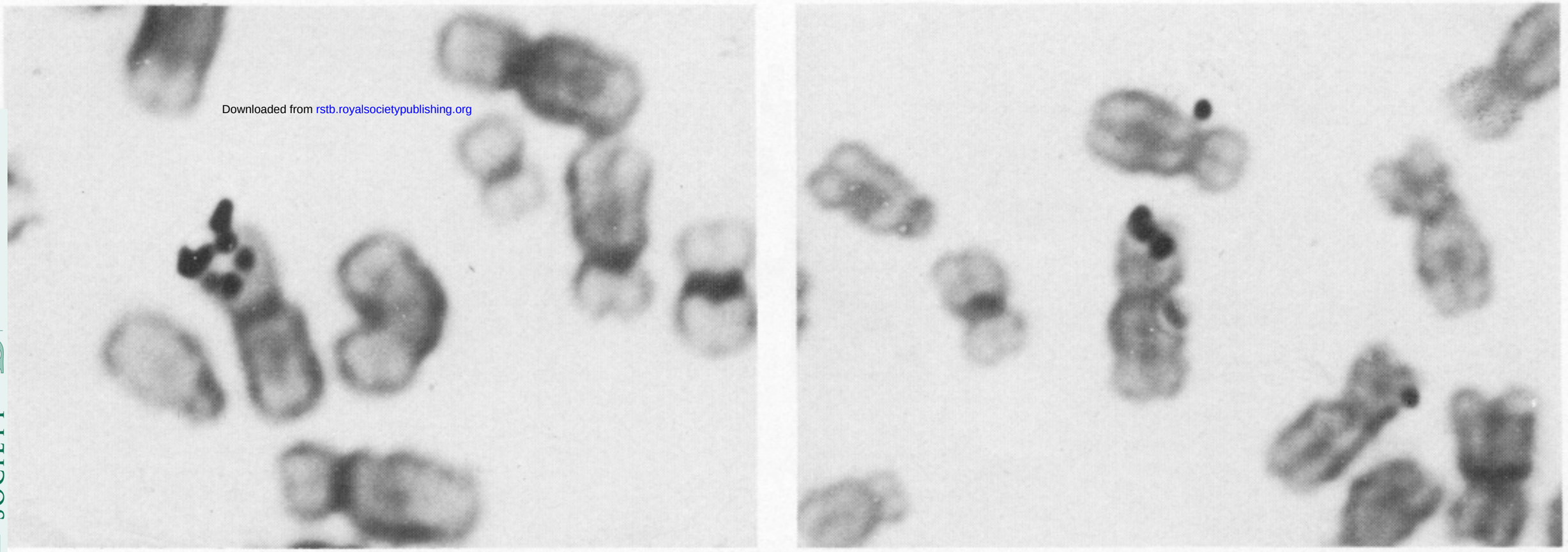

FIGURE 2. *In situ* hybridization of Y-specific probe GMGY10 to the distal end of the short arm of one X-chromosome in an X-Y interchange male.

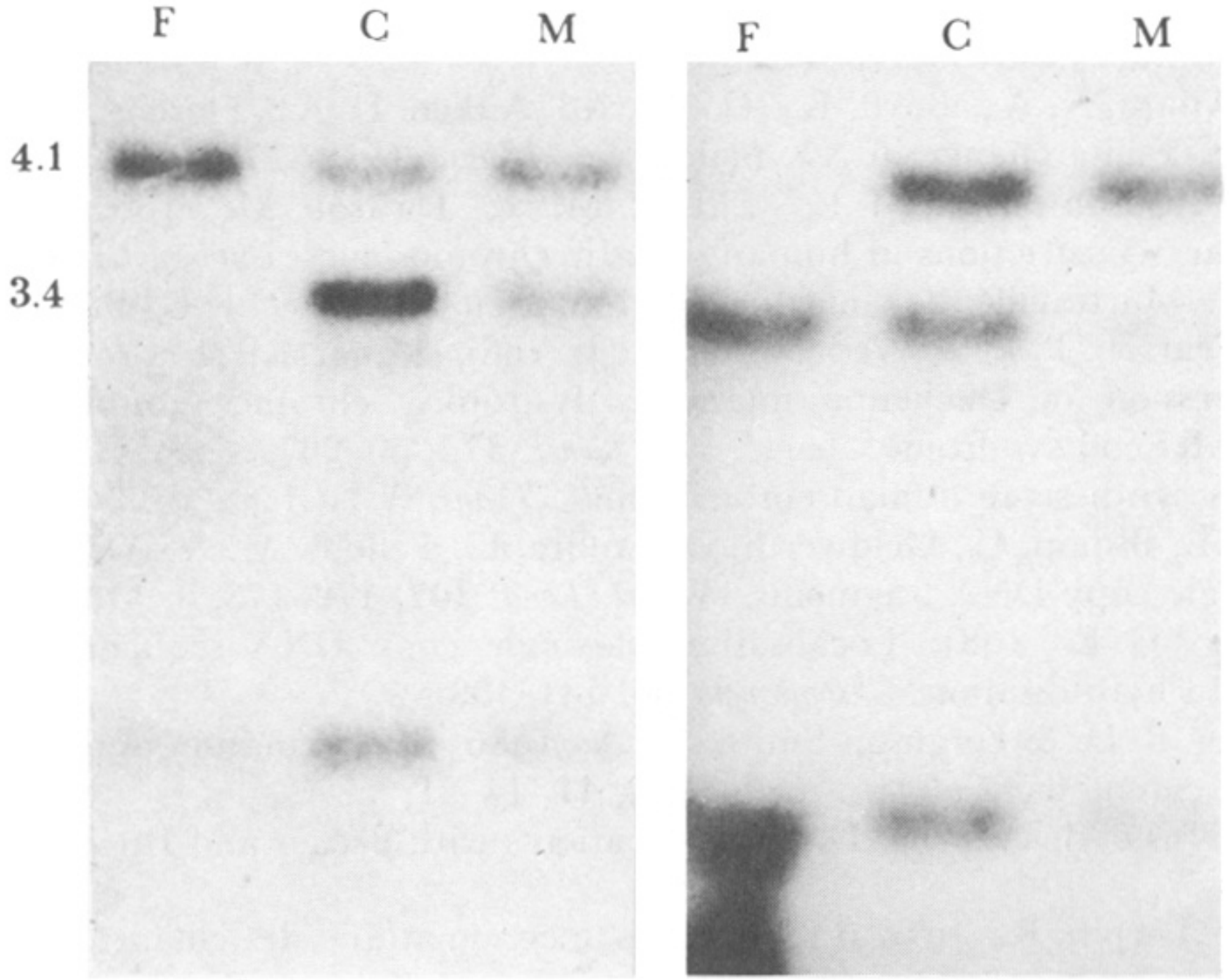

FIGURE 3. Southern analysis of *Msp*I RFLPS recognized by probe D21S15 in two Down's syndrome families. Maternal non-disjunction is indicated in both cases by the increased intensity of the band produced by two maternal chromosomes 21. (F, father; C, Down's syndrome child; M, mother).

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