Figure 5 Mitotic delay evaluated in cells harvested two hours after irradiation with 25-100 cGy x rays (A) or calicheamycin treatment (B). The mitotic fraction in treated samples was expressed as a percentage of the mitotic fraction in the same treated cultures.

Figure 6 Percentage of G₂/M phase cells in untreated and x irradiated cultures harvested 24 hours after treatment. DNA content was evaluated by propidium iodide staining and cytofluorimetry.

detected.⁷⁻⁹ Recently, Stumm *et al.*¹⁰ using a three colour chromosome painting test, found an increased translocation frequency in NBS heterozygotes. In our case, a consistent number of chromosome aberrations in the parents confirms possible chromosome instability in heterozygotes for NBS mutations. Since the chromosomal instabil-

ity is one of the leading causes for developing a malignancy, our data are compatible with the suggestion that NBS heterozygotes could also have an increased cancer risk,¹¹ although no malignancy has yet occurred in our patient's parents.

The parents of our patient, heterozygous for the same mutation, are apparently non-consanguineous and were born in two villages 900 km apart. This may suggest a high frequency of the new mutation in North African populations as reported for 657del5 in eastern Europe.

Our data on DNA damage inducing agents indicate a close similarity between NBS and AT cells in the G₂ chromosomal response after irradiation. This is in agreement with the few published data available on the radiation sensitivity of LCL established from NBS patients.¹²⁻¹³ A higher number of chromatid aberrations in AT and NBS cells, if compared with normal cells, might arise as a result of DNA repair defect or checkpoint dysfunction allowing damaged cells to proceed from G₂ to mitosis. The analysis of G₂ checkpoint activation, as evaluated by cell inhibition to entry to mitosis indicates that after irradiation both NBS and AT cells are less delayed than normal cells. Interestingly, this effect has not been observed in another four NBS lines with either the 657del5 or private mutations showing an intermediate response between normal and AT cells.^{2,12-13} We found such an intermediate response in our NBS cell line after incubation with calicheamycin and this seems to indicate a more specific sensitivity in checkpoint activation of these cells, compared to AT ones, in respect to this specific kind of damage. According to Carney *et al.*¹⁴ and Petrini *et al.*¹⁵ nibrin, the product of the *NBS1* gene, is a member of the hMre11/hRad50 complex involved in dsb repair, by transducing a signal originating from the sites of DNA damage. Moreover, a role of this complex in cell cycle checkpoint activity has been suggested.¹⁵⁻¹⁶ According to our and other published data, the relationship between DNA damage and cell cycle delay is not so straightforward. In fact, in spite of their similar chromosomal radiosensitivity, NBS and AT display strong differences in the percentage of G₂ accumulated cells after irradiation.^{12-13,17} Ionising radiation induced accumulation of p53 and p21, as evaluated at two and four hours after 400 cGy x rays, appears in this NBS line to be similar to that observed in normal cells. These observations are in close agreement with recent reports¹⁷⁻¹⁹ showing that NBS cells, from this point of view, are more similar to normal cells than to AT ones. In conclusion, our results confirm that the radiation mediated response is not identical in NBS and AT. In particular, the cell cycle response of NBS clearly differs from that of AT cells.

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P MARASCHIO*
C DANESINO*
A ANTOCCIA†
R RICORDY‡
C TANZARELLA§
R VARON¶
A REIS**
D BESANA††
A GUALA‡‡
L TIEPOLO*

*Biologia Generale e Genetica Medica, Università di Pavia, Italy

†Dipartimento di Genetica e Biologia Molecolare, Università "La Sapienza", Roma, Italy

‡Centro Genetica Evoluzionistica, CNR, Roma, Italy

§Dipartimento di Biologia, Università "Roma Tre", Roma, Italy

¶Institut für Umangenetik, Humboldt Universität, Berlin, Germany

**Molecular Genetics and Gene Mapping Centre, Max-Delbrueck-Centre, Berlin, Germany

††Neuropsychiatria Infantile, Ospedale C Arrigo, Alessandria, Italy

‡‡Divisione di Pediatria Ospedale S Andrea, Vercelli, Italy

Correspondence to: Dr Maraschio, Via Forlanini 14, 27100 Pavia, Italy, Marasc@unipv.it

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Molecular cytogenetic characterisation of a complex 46,XY,t(7;8;11;13) chromosome rearrangement in a patient with Moebius syndrome

EDITOR—Carriers of de novo balanced reciprocal translocations and inversions have an increased risk of approximately 6% for developing multiple congenital abnormalities (MCA) and/or mental retardation (MR), compared to a 2-3% risk overall in newborn populations.^{1,2} Cytogenetically cryptic deletions or physical disruption or inactivation of a gene(s) in one or both breakpoint regions may account for the observed phenotypes.^{3,4} It seems plausible to assume that the risk for MCA/MR may be even higher in carriers of de novo complex chromosome rearrangements (CCRs), which involve at least three different chromosomes and breakpoint regions. Extreme cases involving up to seven chromosomes and 10 breakpoints have been described.⁵⁻⁷ Indeed, most reported CCRs are associated with MCA/MR.^{8,9} In addition, they have been found in infertile men¹⁰ and in women suffering from multiple miscarriages.^{11,12}

The complex nature of CCRs renders karyotype interpretation by classical chromosome banding alone difficult. In many cases fluorescence in situ hybridisation (FISH) will be the best method to delineate the underlying chromosome rearrangements.^{7,13} Here we have applied conventional FISH with chromosome painting probes and region specific large insert clones, comparative genomic hybridisation (CGH),^{14,15} and spectral karyotyping (SKY)^{16,17} to an apparently balanced and very complex rearrangement in a profoundly retarded patient with Moebius syndrome (MBS, MIM 157900).¹⁸ MBS is characterised by congenital paralysis of the seventh cranial nerve leading to facial diplegia.¹⁹ Other cranial nerves may also be affected. In addition, orofacial and limb malformations, defects of the musculoskeletal system, and MR may occur.

This patient, who has classical Moebius syndrome, has been reported previously.⁴ He was the third child born to a 35 year old father and a 32 year old mother. Because of paresis of the facial muscles as a newborn, he had feeding problems owing to inefficient sucking and swallowing. A more detailed examination at 17 years showed moderate to severe MR and deficient language development. At this time, his height was 1.56 m and his weight 39.5 kg (<3rd centile). Other clinical features included ptosis, blepharophthalmosis, atrophy of the optic nerve, and severe amblyopia. His mandible and facial muscles were hypoplastic. His metacarpals and metatarsals were short, limiting the mobility of his hands and feet. The pectoral muscles were hypoplastic.

Q band analysis²⁰ showed an apparently balanced de novo rearrangement between chromosomes 7, 8, 11, and 13. Breakpoints were assigned to chromosome bands 7q21.1, 8q21.3, 11p14.3, and 13q21.2 (fig 1). CGH did not detect any chromosomal imbalances (sensitivity: gain or loss of >10 Mb DNA) in the patient's karyotype (data not shown). Since an unambiguous interpretation of the rearranged karyotype was not possible by classical chromosome banding analysis, we applied chromosome specific and region specific FISH probes. A schematic representation of our molecular cytogenetic mapping results is shown in fig 2A.

Simultaneous visualisation of all 24 human chromosomes in different colours by SKY was very beneficial in dissecting the patient's karyotype (fig 2B). It confirmed the involvement of chromosomes 7, 8, and 13 in the CCR. The short derivative chromosome 7 was painted in its entirety by the chromosome 7 library. The material distal to 7q21 was translocated onto the der(8) and the der(13). The segment of the der(8) distal to 8q21 was translocated onto der(13). Inversely, the distal long arm 13q21-qter was moved to the der(8). Thus, the der(8) carried additional material from both chromosomes 7q and 13q, whereas the der(13) gained material from both chromosomes 7q and 8q. The chromosome 11 rearrangement, which had been suspected after Q banding, could not be characterised further by SKY. Some metaphases suggested a small insertion into 11p (data not

Figure 1 Q(FH) banded karyotype of a CCR with MBS. Breakpoints (arrowheads) were assigned to chromosomes 7q21.1, 8q21.3, 11p14.3, and 13q21.1.

Figure 2 Molecular cytogenetic characterisation of a CCR associated with MBS. (A) Ideograms of rearranged chromosomes. On the left, the normal chromosomes are displayed, on the right, the derivative chromosomes. Arrowheads indicate the position (or presumed position) of the breakpoints. (B) Spectral karyotype showing the der(7), der(8), and der(13). (C) Metaphase spread hybridised with biotinylated chromosome 13 (green) and digoxigenated chromosome 7 (red) DNA libraries. (D) Metaphase spread hybridised with chromosome 8 (green) and chromosome 11 (red) DNA libraries.

Table 1 Localisation of CEPH YACs on the MBS patient's chromosomes

YAC	Size (kb)	STS	cMpter	Patient's chromosomes	
819b5	1200	D3S3548/D3S3514	149	3q21	3q21
930a7	1500	D3S1292/D3S3514	149	3q21	3q21
754c8	1690	D3S3713	150	3q21	3q21
941d9	1790	D7S524/D7S2417	98/99	7q21	der(11)
690h8	1280	D7S554	110	7q21-22	der(13)
808f3	1790	D7S647	112	7q21-22	der(13)
929a10	430	D7S480/D7S685	127/129	7q31	der(13)
965c12	160	D7S550	181	7q36	der(7)
962g12	1520	D8S544	81	8q12-13	der(8)
953h7	1600	D8S279	90	8q13	der(8)
662e12	ND	D8S521	115	8q21.3-22	der(11)
940h5	1450	D11S1794/D11S1315	22/23	11p15	der(11)
964f4	1330	D11S1359	33	11p15	der(11)
942b11	1790	D11S904	37	11p14	der(11)
765a11	1780	D11S1322	42	11p13-14	der(11)
882b4	1260	D13S1273/D13S172	49/50	13q21-22	der(8) der(13)

cMpter = distance in centimorgans from the short arm telomere. ND = not determined.

shown); however, the chromosomal origin of the inserted material could not be identified. No other chromosome showed detectable abnormalities by SKY.

To overcome the decreased resolution of SKY compared with that of conventional FISH, we performed two colour FISH experiments with DNA libraries of chromosomes 7 and 13 (fig 2C) and of chromosomes 8 and 11 (fig 2D), respectively. In addition to the der(8) and the der(13) detected by SKY, we found an insertion of chromosome 8 material into chromosome 11p14.3 in all metaphases analysed (fig 2D). Sometimes the chromosome 11 paint produced a weak hybridisation signal on the long arm of the der(8) (fig 2D), indicating a reciprocal exchange event between the der(8) and the der(11) (fig 2A).

To FISH map this CCR in fine detail, we selected region specific non-chimeric clones from our standard set of, so far, >2000 cytogenetically and genetically anchored CEPH YACs. These YACs, on average located at 3 cM intervals spaced over the entire chromosome complement, can be adapted with considerable flexibility to the study of chromosome breakpoints.^{4,21} YACs 690h8, 808f3, and 929a10 mapped to the normal chromosome 7q21-31 and to der(13) (table 1). This implies that the proximal part of 7q21-qter, which is missing on the der(7), was translocated onto der(13), whereas the distal part, 7q31-36, was presumably translocated onto der(8). Since the subtelomeric YAC 965c12 showed signals on both the normal and the derivative chromosome 7, but not on der(8) or on der(13), the chromosome 7q end is retained on the der(7). Interestingly, YAC 941d9 from chromosome 7q21 hybridised to the short arm of the der(11). This was not seen by SKY or chromosome painting probes. Taken together, our results indicate that material from the distal long arm of der(7) was relocated to three different derivative chromosomes, the der(8), der(11), and der(13), involving at least four breakpoints on chromosome 7 (fig 2A). The chromosome 8 material inserted into 11p is highlighted by YAC 662e12 from 8q21.3 (table 1). Thus, the small 11p insertion contains sequences from both chromosomes 7 and 8 (fig 2A). YACs 962g12 and 953h7 map proximal to the 8q21.3 breakpoint and, therefore, hybridise to both the normal and the der(8). YACs 940h5, 964f4, 942b11, and 765a11 gave signals on the normal chromosome 11p13-15 and on the der(11) (table 1). The breakpoint region on chromosome 13q21.2 has been studied previously.⁴ The breakpoint spanning YAC 882b4 from 13q21-22 produced a split signal on both the der(13) and der(8).

Autosomal dominant MBS has been linked to markers on 3q21 in a large Dutch family (MBS2, MIM 601471).^{18,22} To analyse whether rearrangements involving the MBS2 locus may be responsible for the phenotype in our patient, a microdissected DNA library specific for the long arm of

chromosome 3,²³ as well as YACs 819b5, 930a7, and 754c8 from the critical region on chromosome 3q21 (table 1) were hybridised to the patient's chromosomes. No structural abnormalities were detected on chromosome 3.

Collectively, molecular cytogenetics showed at least four breakpoints on chromosome 7, two each on chromosomes 8 and 11 and one on chromosome 13. To our knowledge, this case represents one of the most complex constitutional chromosome rearrangements reported: 46,XY,t(7;8;11;13)(7pter→7q21.1::7q36→7qter;8pter→8q21.3::11p14.3→11p14.3::7q31→7q36::13q21.2→13qter;11pter→11p14.3::8q21.3→8q21.3::7q21.1→7q21.1::11p14.3→11qter;13pter→13q21.2::7q21.1→7q31::8q21.3→8qter).

Classical Q, G, or R banding is the screening method of choice for numerical and structural chromosomal abnormalities. However, in some instances, for example, subtelomeric²⁴ and other cytogenetically cryptic rearrangements^{4,25} or CCRs,^{7,13} chromosome banding techniques alone may not allow an exact karyotype interpretation and correlation between genotype and phenotype. This is why FISH has become an important accessory technique in clinical cytogenetics. In this study, we have used state of the art molecular cytogenetic tools, most importantly SKY and a panel of region specific YACs, to elucidate a very complex chromosome rearrangement, which occurred as a de novo constitutional aberration in a MBS patient. Of course molecular cytogenetics cannot replace classical chromosome banding, but it certainly is a very useful complementary strategy.

Q banding identified four rearranged chromosomes. SKY was able to visualise this CCR in a single hybridisation experiment and confirmed the involvement of three chromosomes, 7, 8, and 13. However, it did not reliably detect the insertion on chromosome 11p, which contains sequences homologous to YAC 941d9 from chromosome 7 and YAC 662e12 from chromosome 8. Evidently, this insertion, which altogether extends over at least 2 Mb, is below the limit of DNA resolution by SKY. Under optimal experimental conditions, which may be hard to achieve in a routine laboratory and for all chromosomal regions, the resolution of SKY is estimated to be 1-2 Mb (from a single chromosome).^{16,17} We conclude that SKY is a very straightforward and fast screening technique for identifying (most) chromosomes involved in a given CCR. However, subsequent FISH experiments with chromosome painting probes and region specific YACs are crucial for exact karyotype interpretation. In particular, our standard set of cytogenetically and genetically anchored YAC probes allows the rapid and accurate characterisation of breakpoint regions of interest and of smaller chromosome segments involved in CCRs.

Identification of YAC clones spanning disease associated chromosome breakpoints will greatly facilitate positional cloning projects in cases where the phenotype is caused by disruption of a relevant disease gene in the breakpoint region. We have suggested previously that chromosome 13 may harbour a gene for MBS.⁴ In addition to the patient described here, there is a t(1;13)(p34;q13) translocation segregating in a family with MBS²⁶ and a de novo deletion of 13q12.2 in a girl with MBS.²⁷ Although these chromosome rearrangements are located proximal to the 13q21 breakpoint region of our patient, it is still possible that the same gene is affected. The published cases have not been analysed by molecular cytogenetic techniques, and breakpoint localisation by chromosome banding alone may be inaccurate by several bands. A second locus for MBS has been linked to chromosome 3q21.²² However, since this region did not show detectable rearrangements in the CCR studied, it is not very likely that the MBS2 gene on chromosome 3 is affected. MBS is a very heterogeneous clinical syndrome. In a family with autosomal dominant MBS, both the MBS1 locus on

chromosome 13q12.2-q13 and the *MBS2* locus on chromosome 3q21 have been excluded.²² Therefore, a cytogenetically cryptic deletion or disruption of a gene in a breakpoint region other than 13q21 may be responsible for the MCA/MR phenotype of our patient. In this context, it is interesting to note that an unrelated MBS patient with a breakpoint on chromosome 11p13 has been described.²⁸ Therefore, the 11p insertion may be the chromosome rearrangement underlying the disease in our patient. Of course, we also cannot exclude the possibility that MBS/MR may be associated with the observed CCR by chance or be caused by a non-genetic mechanism.¹⁹

More than 100 CCRs have been described so far. With the advances in FISH and multicolour techniques, it has become possible for the first time to study even very complex cases in detail. Overall many CCRs may be even more complicated than suggested by classical chromosome banding. Interestingly, CCR breakpoints are not randomly distributed throughout the entire chromosomal complement, but appear to be clustered in certain chromosome regions. In particular, the 7q21, 8q21, and 11p14 regions, which are involved in our patient's CCR, seem to be prone to chromosome breakage. In a recent survey of 100 CCRs, nine breakpoints were found in 18q21; seven each in 3q13 and 7q22; six in 5p13, 7q11, and 8q22; five in 1p22, 1q42, 3p13, 7q21, 9p24, 11q14, and 21q21; four in 1p13, 2q13, 2q21, 2q33, 3p11, 3q26, 7q31, 8p23, 8q13, 8q24, 11p15, 13q32, 21q11, and 21q22; and three in 1q21, 1q32, 2q22, 2q23, 3p23, 5p14, 5q15, 6p21, 6q27, 7p14, 7q36, 8q21, 9q22, 9q32, 10p12, 10q11, 10q22, 11p14, 11p13, 11q13, 11q21, 12p13, 12q22, 12q24, 13q14, 15q22, and 21p11.⁸ This breakpoint clustering is probably not the result of ascertainment bias, but suggests the existence of chromosomal "hot spots" involved in complex rearrangements. The molecular mechanism(s) underlying frequent breakage remains to be elucidated.

A significant proportion of disease associated chromosome breakpoints involve submicroscopic deletions of several megabases of DNA.⁴⁻²⁵ Considering the complex nature of the CCR described, excluding microdeletions in one or several of the breakpoint regions involved by high resolution FISH mapping alone will be a very time consuming or even impossible task. Only miniaturisation and automation of techniques will allow one to screen the entire genome in a reasonably short time for cytogenetically cryptic (unbalanced) rearrangements. However, with the development of CGH of patient and reference DNAs on microarray DNA chips,²⁹⁻³⁰ this goal seems to be within reach and a new era in the analysis of chromosome disorders has begun.

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G BORCK*
J WIRTH*
T HARDT*
H TÖNNIES†
K BRØNDUM-NIELSEN‡
M BUGGE§
N TOMMERUP§
H-G NOTHWANG*
H-H ROPERS*
T HAAF*

*Max Planck Institute of Molecular Genetics, Ihnestrasse 73, 14195 Berlin, Germany

†Institute of Human Genetics, Humboldt University, Berlin, Germany

‡J F Kennedy Institute, Glostrup, Denmark

§Department of Medical Genetics, IMBG, Copenhagen, Denmark

Correspondence to: Dr Haaf, Haaf@molgen.mpg.de.

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Use of a set of highly polymorphic minisatellite probes for the identification of cryptic 1p36.3 deletions in a large collection of patients with idiopathic mental retardation

EDITOR—Mental retardation is a component of a large number of syndromes, most of which qualify individually as rare genetic diseases. Altogether, mental retardation affects 2-3% of the population and is unexplained in 40% of cases. According to Knight *et al.*,¹ subtle telomeric chromosomal rearrangements are responsible for approximately 1% of unexplained mental retardation (with the proportion being highest, 7.4%, in the subclass of unexplained moderate to severe mental retardation). The identification of the genes responsible will require the precise delimitation of minimum deletion regions, which relies upon the collection of a large number of cases. Because of the low expected frequency of each telomeric deletion in mental retardation, the procedure to be applied should allow the screening of many patients at a low cost.

It is not yet clear whether all chromosome ends are associated with mental retardation syndromes with similar frequency. Distal chromosome 1p36 deletions were initially detected cytogenetically because of an associated segmental imbalance.²⁻⁵ This syndrome results from both interstitial and terminal deletions of varying sizes and different breakpoints⁶ and the severity of the phenotype is related to the extent of the deletion.⁷ Clinical examination can efficiently detect a large proportion of cases, so that the number of reports of 1p36 deletions has increased significantly in the past few years which may give the false impression that this is a relatively frequent syndrome.

We show here how highly polymorphic minisatellites located within a short region can provide an efficient pre-screening of samples without the need for parental samples at the initial stage. The procedure was tested here using 1p36 minisatellites and provides an estimate of the frequency of 1p36 deletions in mentally retarded patients. A selection of five highly polymorphic minisatellite probes was used to search for 1p36.3 deletions in a collection of 567 patients with mental retardation. Three patients showing a single allele at all five loci were identified and in each case segmental 1p36 aneusomy was confirmed by *in situ* hybridisation. The physical extent of each deletion was determined by analysis of additional markers. In two cases, the parental origin of the deletion could be determined, one is maternal and the other is paternal. Analysis of band intensity of samples showing a single allele at two or more contiguous minisatellite loci ruled out the possibility of shorter deletion in this collection of patients. These data and earlier investigations suggest that 1p36.3 deletions account for as much as 0.5-0.7% (96% confidence interval 0.15-1.2%) of idiopathic mental retardation.

All participating families gave informed consent to the search for a genetic abnormality which might explain the condition of their child. Most DNA samples (469/567) were initially collected and extracted in the course of a search for the fragile X. All patients showed a normal routine karyotype.

DNA was extracted from blood collected on EDTA essentially as described by Jeanpierre.⁸ Five µg of DNA

were digested with *AluI* (Boehringer Mannheim), electrophoresed through a 1% agarose gel, and transferred to nylon membranes (Nytran+, Schleicher and Shuell) under vacuum (Pharmacia Biotech).

Minisatellite DNA inserts were [³²P]dCTP labelled by random priming⁹ and hybridisation was done at 65°C as previously described.¹⁰ The minisatellites used in this study (CEB66, B4, CEB42, CEB108, CEB15, CEB88, CEB37, UPS2, CEB20, CEB55, and CEB121) were isolated essentially as previously described.¹⁰⁻¹³ CEB66, B4, and CEB42 minisatellites are located at 13q34, Xpter, and 8q24 respectively. CEB66 and B4 are monomorphic and were used when necessary as controls for DNA quantification. The highly polymorphic CEB42 probe was used to confirm parentage. The other loci have been previously assigned to 1p36.3 by FISH and segregation analysis.¹²⁻¹⁵ All probes used in this study have been used in linkage mapping in the CEPH families and no evidence for *de novo* deletion, trisomy, or UPD in the DNA extracted from lymphoblastoid cell lines (more than 500 subjects) was found.

Microsatellites were purchased from Research Genetics. One primer was kinased with γ[³³P]dATP (Isotopchim) as recommended by the manufacturer (kinase from New England Biolabs). PCR reactions were performed as previously described¹⁶ using a Perkin-Elmer GeneAmp PCR system 9600. PCR products were separated on 6% polyacrylamide/urea gels. The gel was dried after transfer onto a Whatman 3MM paper and exposed to an RX Fuji film.

Segregation data in the CEPH families were managed using the GENBASE software developed by Jean-Marc Sebaoun at CEPH.¹⁷ Analyses were done using the CRIMAP version 2.4 package.¹⁸ Minisatellites were typed on the 40 CEPH families by Southern blotting. Meiotic breakpoint analysis was further assisted by the software described by Attwood and Povey.¹⁹ Microsatellite genotyping data on the eight CEPH families were recovered from the CEPH database at <http://www.cephb.fr>. Microsatellite typings on the remaining 32 CEPH families were done to increase the mapping resolution. In order to reduce typing effort, only key recombinant subjects were typed, together with the two parents and appropriate grandparents as outlined in Cox *et al.*²⁰ All genotyping data generated in the course of this project can be recovered from CEPH at <http://www.cephb.fr/>.

Chromosome preparations were made from peripheral blood samples or lymphoblast cultures by conventional methods. Karyotyping with RBG banding was performed at the 550-850 band level according to standard methods. Alternatively, high resolution R banding was obtained by introduction of 5-bromodeoxyuridine (BrdU) for 5½ hours in cell cultures after thymidine synchronisation.²¹

Cosmid DNA was labelled with digoxigenin 11dUTP using the Dig-Nick translation kit from Boehringer Mannheim Inc. One hundred ng of the resulting digoxigeninylated DNA were preannealed with 2.5 µg of cot I DNA (Gibco BRL) in a total volume of 10 µl hybridisation mix (50% formamide, 2 × SSC, 10% dextran sulphate) for 30 minutes at 37°C. Hybridisation to the previously denatured chromosomes was done overnight at 37°C. Post hybridisation wash was carried out at 72°C in 2 × SSC for five minutes. Probe detection was performed with antidigoxin conjugated fluorescein isothiocyanate (FITC, Sigma). Slides were mounted in antifade solution (Vectashield, Vector Laboratories) containing 4', 6-diamidino-2-phenyl indole (DAPI) as a counterstain.