

# Characterization of the Supernumerary Chromosome in Cat Eye Syndrome

HEATHER E. McDERMID,\* ALESSANDRA M. V. DUNCAN, KLAUS R. BRASCH,† JEANNETTE J. A. HOLDEN, ELLEN MAGENIS, ROBERT SHEEHY, JOHN BURN, NATALINE KARDON, BERNARD NOEL, ALBERT SCHINZEL, IKUKO TESHIMA, BRADLEY N. WHITE‡

Most individuals with cat eye syndrome (CES) have a supernumerary bisatellited chromosome which, on the basis of cytogenetic evidence, has been reported to originate from either chromosome 13 or 22. To resolve this question, a single-copy DNA probe, D22S9, was isolated and localized to 22q11 by in situ hybridization to metaphase chromosomes. The number of copies of this sequence was determined in CES patients by means of Southern blots and densitometry analysis of autoradiographs. In patients with the supernumerary chromosome, four copies were found, whereas in one patient with a duplication of part of chromosome 22, there were three copies. Therefore, the syndrome results from the presence of either three or four copies of DNA sequences from 22q11; there is no evidence that sequences from other chromosomes are involved. This work demonstrates how DNA sequence dosage analysis can be used to study genetic disorders that are not readily amenable to standard cytogenetic analysis.

**I**N CAT EYE SYNDROME (CES), A SMALL supernumerary chromosome is associated with various malformations, including ocular colobomata, anal and cardiac defects, and mental retardation (1–3). Each associated feature is relatively common and none is necessarily present in every instance of CES. Although the extra chromosome is too small to identify with chromosomal banding, cytogenetic evidence and subject phenotype suggest that it may be derived from chro-

mosome 13, chromosome 22, or both (1, 2). We have analyzed the number of copies of a DNA sequence located on chromosome 22 to resolve the question. A recombinant DNA library was constructed in the  $\lambda$  phage vector L47.1 with DNA isolated from a chromosome fraction enriched for chromosome 22 (4). A 1.8-kilobase (kb) Eco RI–Hind III fragment (D22S9) was subcloned into pUC8 and localized by in situ hybridization to 22q11 (Fig. 1a). This localization

was confirmed by quantitative analysis of DNA hybridization in Southern blots that showed the presence of D22S9 in three copies in DNA from cells trisomic for 22pter→22q11 (PT2) (5), but in two copies in normal individuals (N) (Fig. 2, a and b). D22S9 hybridized to both chromosome 22 and the supernumerary bisatellited chromosome in metaphase spreads from an individual with CES (Fig. 1b, subject P.O.). Of the 306 silver grains scored from 150 cells examined, 8.5 percent were located over the two chromosome 22 homologues and 6.9 percent were located over the single bisatellited marker, indicating the presence of one or two copies of D22S9 on the marker.

The DNA probe D22S9 was hybridized to DNA's from random, normal Canadians (N), cell lines partially trisomic for chromosome 22 (5), and eight other human subjects (6). The copy number of D22S9 was determined by analyzing the ratios of the hybridization signal from D22S9 to the chromosome 11 probe e9-12/1 (7). Two sets of experiments with three and five replicates per blot were performed and the results pooled. Six CES patients with a bisatellited marker (S2, S5, O.L., K.C., I.G., and M.S.) gave similar results (Fig. 2a and Table 1). When the chromosome 11 standard bands were of equal intensity, the D22S9 band increased in intensity such that CES > partially trisomic > normal. Densitometric

Table 1. Determination of copy number of the D22S9 sequence in partial trisomy 22 cells (PT1 and PT2) and in eight subjects. A basic data set consisted of two lanes of an autoradiogram (Fig. 2), one lane containing normal DNA and the other containing either partial trisomy-22 DNA or subject DNA. The ratio between the signal from D22S9 and that from e9-12/1 was determined for each lane. These standardized measures of D22S9 hybridization were used to form a second ratio, which was that of the subject or partial trisomy 22 to the normal. This second ratio allowed pooling of information from multiple Southern blots. A ratio of 1.5 is expected for three copies of D22S9 and a ratio of 2.0 for four copies. Sample size (*n*) refers to the number of replicate lane sets used to calculate each final ratio. PT1 and PT2 represent the data from two partial trisomy 22 cell lines, which, because they were homogeneous, were pooled. The data points for each subject were compared with the 41 data points for the partial trisomy cell line by a two-tailed Wilcoxon rank-sum test (14). The null hypothesis in each case was that the data points of each subject and those of the partial trisomy were taken from the same underlying distribution. Levels of significance are given here for individual tests and in the text for the overall comparison.

Source of DNA	<i>n</i>	Normalized signal ratios from D22S9 and e9-12/1 ( $\bar{X} \pm \text{SEM}$ )	Individual test level of significance
PT1 and PT2	41	1.58 ± 0.04	
S2	8	2.11 ± 0.10	<0.001
S5	8	1.90 ± 0.14	0.008
O.L.	8	2.26 ± 0.14	<0.001
K.C.	8	2.29 ± 0.08	<0.001
I.G.	8	2.26 ± 0.15	<0.001
M.S.	8	2.69 ± 0.16	<0.001
L.W.	8	1.52 ± 0.07	0.497
J.B.	3	0.89 ± 0.10	0.002

Table 2. Ratios of the signal intensity of the Taq I alleles identified by D22S9 for partial trisomy 22 cells (PT1 and PT2) and four subjects. The ratio of the multicopy allele to the single allele was determined for each lane of the autoradiogram of the partial trisomies and the four subjects and normalized with the associated control ratio. The expected normalized allele ratio is 2 for three copies and 3 for an individual with four copies. Probabilities were determined as in Table 1, except that a one-tailed procedure was used.

Source of DNA	<i>n</i>	Normalized allele ratio ( $\bar{X} \pm \text{SEM}$ )	Individual test level of significance
PT1 and PT2	24	2.34 ± 0.08	
K.C.	10	3.19 ± 0.28	0.007
I.G.	10	3.37 ± 0.25	<0.001
M.S.	10	3.25 ± 0.29	<0.001
J.B.	5	1.14 ± 0.17	<0.001

H. E. McDermid, K. R. Brasch, B. N. White, Department of Biology, Queen's University, Kingston, Ontario K7L 3N6, Canada.  
A. M. V. Duncan and J. J. A. Holden, Departments of Biology and Paediatrics, Queen's University, Kingston, Ontario K7L 3N6, Canada.  
E. Magenis and R. Sheehy, Department of Medical Genetics, Oregon Health Sciences University, Portland, OR 97201.  
J. Burn, Paediatric Genetics Unit, Institute of Child Health, London, WC1N1EH, England.  
N. Kardon, Booth Memorial Medical Center, Flushing, NY 11355.  
B. Noel, Laboratoire de Cytogénétique, Centre Hospitalier, Chambéry, France.  
A. Schinzel, Institute of Medical Genetics, University of Zurich, Zurich, Switzerland.  
I. Teshima, Genetics Department, Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada.

\*Present address: Children's Hospital of Philadelphia, Philadelphia, PA 19104.

†Present address: Faculty of Natural Sciences, University of Tulsa, Tulsa, OK 74104.

‡To whom correspondence should be addressed.

scans of the signals confirmed this, with the partially trisomic DNA approximately 1.5 times as intense as normal and CES DNA giving signals twice as intense as normal (Table 1). The Wilcoxon test was used to examine the null hypothesis that the signal ratios for each of the eight subjects were a random sample of partially trisomic values. The eight tests were not independent in that they made use of the same 41 partially trisomic values and, therefore, the overall level of significance was conservatively estimated by use of Bonferroni's inequality (8). This established the individual test probability at 0.00625 (0.05/8). Five of six CES patients with the supernumerary chromosome showed significantly more hybridization than the partial-trisomy DNA, indicating they had at least four copies of the region of chromosome 22 where D22S9 is located (22q11).

Two subjects (L.W. and J.B.) showed no increased hybridization of D22S9 over the partial trisomic DNA's (Fig. 2, b and c). Subject L.W., who has a CES phenotype and an interstitial duplication of chromosome 22, but no supernumerary chromosome, had three copies of the D22S9 region, which was not significantly different from the signal ratio in partially trisomic DNA's. Also, the normalized signal ratio of J.B., who has a bisatellited marker but who is phenotypically normal, was significantly less than that of the partial trisomics, suggesting the presence of two copies (Table 1). Further cytogenetic investigation of the chromosome of J.B. by staining with distamycin A/DAPI has revealed that the extra chromosome is a dicentric 15pter→q11::q11→15pter (9).

A restriction fragment length polymorphism identified by D22S9, with Taq I alleles of 5.8 kb (A-1) and 3.2 kb (A-2), allowed the use of the allele present in one copy as the standard in heterozygotes and eliminated the need to use a nonsynthetic probe. Both partially trisomic cell lines were heterozygous, each showing one allele with increased hybridization. Four subjects (K.C., I.G., M.S., and J.B.) were also heterozygotes. A series of blots comparing replicates each of normal, partially trisomic, and subject DNA (Table 2) showed that three subjects (K.C., I.G., and M.S.) had allele ratios significantly greater than those of the partial trisomics, but were not significantly different from one another, as evaluated by the overall level of significance of 0.0125 (0.05/4). The conclusion that the CES patient DNA's contain at least as many or more copies of D22S9 as partially trisomic DNA was used to justify the one-tailed Wilcoxon test (Table 2). The allele ratios confirm the presence of four copies of

D22S9 in these CES subjects with the supernumerary chromosome, including M.S., who showed a significantly elevated ratio in the previous test (Table 1) and which could have been interpreted as five copies. The

allele ratio of J.B. approached 1, confirming the presence of two copies of D22S9.

We conclude that CES is usually the result of an extra chromosome consisting of a duplication of the DNA between

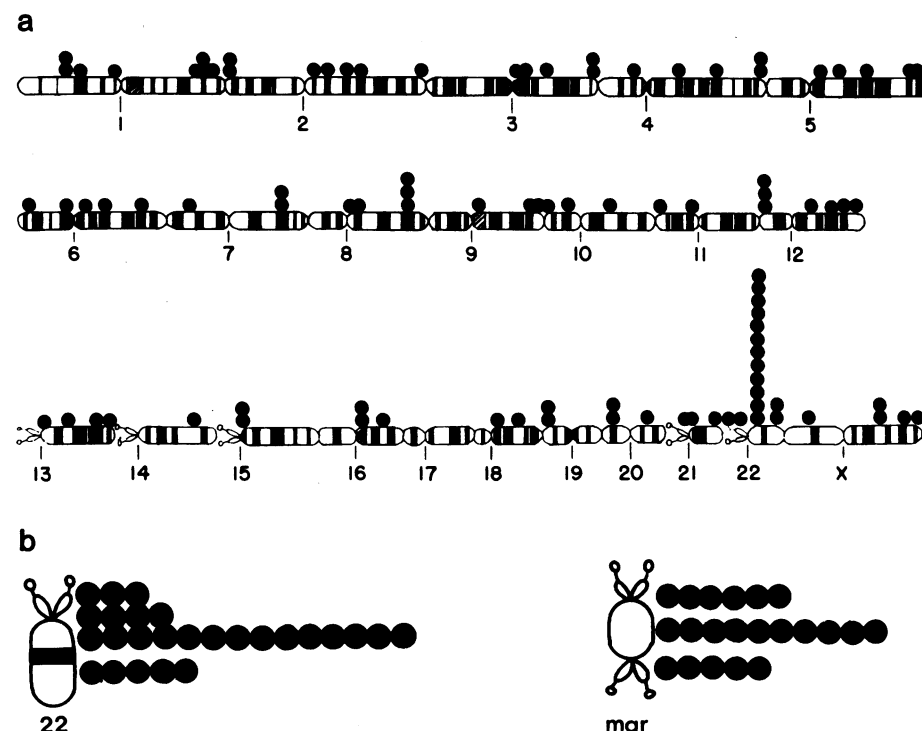
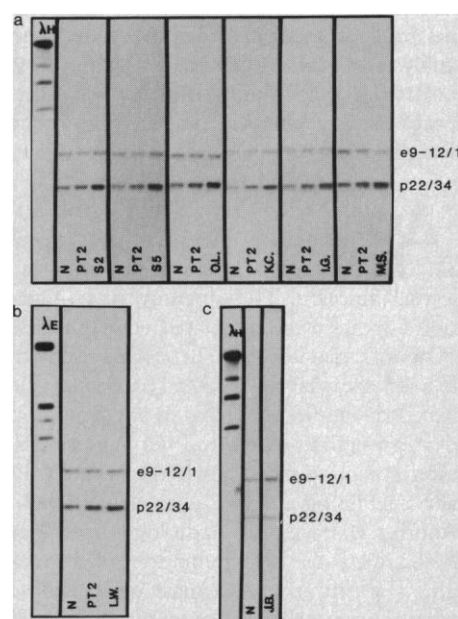


Fig. 1. In situ hybridization localization (12) of D22S9 to 22q11 with a normal subject (a) and to 22q11 and the bisatellited marker chromosome (mar) in a subject (P.O.) with CES (b). Of 100 silver grains scored in (a), 12 were located on band 22q11. There were no more than three grains over any other band. A total of 26 (8.5 percent) and 21 (6.9 percent) of 306 silver grains scored in (b) were observed on the normal 22's and the marker (mar) chromosome, respectively.

Fig. 2. Hybridization of D22S9 (labeled p22/34) and the chromosome 11 probe e9-12/1 to DNA from eight subjects. DNA was obtained from either blood [S2, S5, L.W., M.S., I.G., J.B., and normal controls (N)] or fibroblast cultures (O.L., K.C., and PT2), digested with Eco RI and Hind III and transferred from 1 percent agarose gels to GeneScreen Plus. DNA from subjects to be compared was loaded in replicates of three to five for statistical analysis. To improve transfer, the DNA was nicked by exposing the gels to ultraviolet light for 5 minutes before being denatured. The blots were hybridized in a large volume (40 ml) in freezer bags kept flat with glass plates. Mechanical agitation during the 16- to 24-hour hybridization, as well as occasional manual mixing, ensured continuously even distribution of the probes. Blots were washed first in two changes of  $\times 2$  standard saline citrate (SSC) at 25°C (5 minutes each) and then in three changes of  $\times 2$  SSC-0.2 percent sodium dodecyl sulfate at 65°C (30 minutes each). A Beckman DU-8 spectrophotometer was used to scan the absorbance at 540 nm of each lane of the autoradiograms (13). The area under each peak was determined with a Zeiss MOP-3 digital image analyzer, each value being the average of three separate tracings. The signal ratios (D22S9:e9-12/1) were divided by the associated normal control signal ratio from the same blot for normalization purposes. Ratios of subjects were compared with the Wilcoxon rank-sum test (14). (a) Subjects S2, S5, O.L., K.C., I.G., and M.S.; (b) subject L.W.; (c) subject J.B. Designations  $\lambda$ H and  $\lambda$ E represent  $\lambda$ DNA digested with Hind III and Eco RI, respectively.



22pter→q11, which can be represented as dic(22;22)(q11;q11) or 22pter→q11::q11→22pter (four copies of D22S9), but can also result from an interstitial duplication of the 22q11 region (three copies of D22S9). The latter may explain the few reported CES cases that lack an extra chromosome (10). Our conclusion can extend only to the patients tested, as a random selection of subjects would be impossible because of the small numbers available. However, the selection was made only on the basis of availability, and, although most are from the more severely affected end of the CES spectrum, one (I.G.) was mildly affected and does show four copies of D22S9.

In this study we applied densitometric and statistical analysis of Southern blots to examine genetic conditions that are not amenable to study by standard cytogenetic methods. A second condition for which

probe D22S9 could be useful is DiGeorge syndrome, which is characterized by aplasia of the thymus and parathyroids, and which may be associated with a small deletion at 22q11 (11). A similar approach may prove useful in assessing the chromosomal composition of tumors, since the need to culture such tissue is eliminated.

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## A Neuronal Antigen in the Brains of Alzheimer Patients

BENJAMIN L. WOLOZIN, ALEX PRUCHNICKI, DENNIS W. DICKSON, PETER DAVIES

A monoclonal antibody was prepared against pooled homogenates of brain tissue from patients with Alzheimer's disease. This antibody recognizes an antigen present in much higher concentration in certain brain regions of Alzheimer patients than in normal brain. The antigen appears to be a protein present in neurons involved in the formation of neuritic plaques and neurofibrillary tangles, and in some morphologically normal neurons in sections from Alzheimer brains. Partial purification and Western blot analysis revealed the antigen from Alzheimer brain to be a single protein with a molecular weight of 68,000. Application of the same purification procedure to normal brain tissue results in the detection of small amounts of a protein of lower molecular weight.

**A**LZHEIMER'S DISEASE IS A NEURO-degenerative disorder characterized clinically by progressive loss of intellectual function. This impairment of function appears to be correlated with numbers of neuritic plaques in the neocortex and with loss of presynaptic markers of cholinergic neurons (1). Neuritic plaques are composed of degenerating axons and nerve terminals, often surrounding an amyloid core and usually containing reactive glial elements (2). Another characteristic pathologic feature of Alzheimer's disease, the neurofibrillary tangle, is an intraneuronal mass composed of normal intermediate filaments and paired helical filaments (PHF) with unusual properties (3).

In studying the topographic distribution of plaques and tangles in the brains of

Alzheimer patients, we noted that the lesions occur with high frequency in regions receiving cholinergic innervation from the ventral forebrain (4). This cholinergic cell group appears to be extremely vulnerable to the disease process, and evidence that cholinergic nerve terminals participate in plaque formation has been presented (5). To better define the relation between ventral forebrain cholinergic neurons and the lesions of the Alzheimer brain, we have prepared monoclonal antibodies to homogenates of ventral forebrain tissue taken at autopsy from four patients with Alzheimer's disease. The resulting antibodies were screened on the basis of their ability to differentiate brain tissue from patients with Alzheimer's disease and from normal subjects in both immunohistochemical and immunocytochemical procedures.

Antibodies were initially assayed according to their ability to bind to brain homogenate that had been immobilized onto polyvinyl plates (1 µg per 50-mm diameter well) by drying at 37°C for 1 hour. Antibody binding was detected with peroxidase-conjugated goat antibody to mouse immunoglobulins. Those antibodies that showed greater than a 50% increase or decrease in binding to homogenates of Alzheimer brain relative to normal tissue were studied further. One of these antibodies, Alz-50, is described below.

Initial assays showed that the binding of Alz-50 was highly selective for brain tissue from Alzheimer patients. Figure 1 shows that 0.33 µg of temporal cortex homogenate from Alzheimer patients gave an optical density only slightly lower than 10 µg of temporal cortex homogenate from normal patients. From these data we conclude that the antigen is elevated 15 to 30 times in the temporal cortices of the Alzheimer patients. Alz-50 reactivity was similarly elevated in the nucleus basalis and hippocampus. These areas, cortex, nucleus basalis, and hippocampus, are all known to contain neuritic plaques and neurofibrillary tangles in brains of patients with Alzheimer's disease. Brain areas less affected by the disease, such as caudate, thalamus, or cerebellum demonstrated little or no reactivity.

The immunocytochemistry of Alz-50 on

Departments of Pathology and Neuroscience, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461.