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Molecular analysis of chromosome 11 deletions in aniridia–Wilms tumor syndrome

(catalase gene/somatic cell hybrids/gene mapping/fluorescence-activated cell sorting analysis)

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ABSTRACT **We describe five individuals who have constitutional deletions of the short arm of one chromosome 11, including all or part of the band p13. All of these individuals suffer from aniridia; two have had a Wilms tumor removed. We have established lymphoblastoid cell lines from these and in three cases constructed somatic cell hybrids containing the deleted chromosome 11. Analysis of DNA from the cell lines and hybrids with a cloned cDNA probe has shown that the catalase gene is deleted in four of five patients. The catalase locus must be proximal to the Wilms and aniridia-related loci. We have not detected a deletion of the β-globin or calcitonin genes in any of these individuals; we conclude these genes are likely to be outside the region 11p12-11p15.4. In addition, we have used monoclonal antibodies in fluorescence-activated cell sorting analysis to measure expression in the hybrids of two cell surface markers encoded by genes that map to the short arm of chromosome 11. The genes for both of these are deleted in two individuals but are present in the individual with the smallest deletion.**

Wilms tumor is a nephroblastoma affecting ≈1 in 10,000 children (1). One in 50 children with this condition also has congenital aniridia (absence of iris), which usually leads to blindness (2). Conversely, 1 in 3 individuals with sporadic (nonfamilial) aniridia develops Wilms tumor. Patients with the aniridia–Wilms tumor (AWT) syndrome are usually mentally retarded and often suffer from other urinogenital abnormalities. The majority of AWT patients is heterozygous for a constitutional deletion that always includes at least part of band p13 on the short arm of chromosome 11 (3, 4). Individuals with visible deletions embracing the whole of band 11p13 invariably have aniridia, but only present with Wilms tumor in about half of the cases, suggesting incomplete penetrance (5). In several cases of Wilms tumor without aniridia or obvious deletion, the involvement of chromosome 11 has been demonstrated by the loss of an allele for one or more chromosome 11 short arm markers in tumor tissue relative to heterozygous normal tissue from the same patient (6–9). It appears therefore that in nephroblastoma, as in retinoblastoma (10), there is selection for homozygosity of a defective or absent allele in the course of tumorigenesis. Thus, at the cellular level Wilms tumor is recessive and the observed incomplete penetrance is consistent with a two-hit hypothesis of tumor development (11). The normal product of the "Wilms gene" may function to suppress the action of a second target gene in kidney development. If the activity of the target gene is not counteracted, uncontrolled cell proliferation results. Aniridia, however, may arise by haploid insufficiency, when one of the normal genes is lost.

The availability of overlapping deletions will help in the isolation of these genes and in the construction of a physical linkage map of chromosome 11p. As a first step toward isolating these genes we must identify the genes or DNA deleted in all patients and define the smallest region of overlap. We describe five individuals with aniridia having varying sizes of overlapping deletions and different combinations of congenital abnormalities. Lymphoblastoid lines and somatic cell hybrids were analyzed by using markers previously assigned to chromosome 11p. These include DNA probes for β-globin (HBB), calcitonin (CALC), parathyroid hormone (PTH), and catalase (CAT), the last of which has been shown by enzyme analysis to be reduced in a number of AWT patients with deletions (12). We also followed the expression of the enzyme lactate dehydrogenase A (LDHA) and of two cell surface antigens defined by antibodies F10.44.2 and 163A.5 (13, 14).

MATERIALS AND METHODS

Cell Culture. Peripheral blood lymphocytes (≈2 × 10^6) were cultured with Epstein–Barr virus. Transformed cell lines, obtained within 3–5 weeks, were grown in RPMI 1640 medium supplemented with 5% fetal calf serum, 0.2 international unit of porcine insulin per ml, 1 mM oxaloacetic acid, 0.45 mM sodium pyruvate, and 12.5 mM 3-(*N*-morpholino)-propanesulfonic acid.

Somatic cell hybrids were produced by fusion between the thioguanine-resistant mouse myeloma cell P3-NS1/1-Ag4-1 (15) and human lymphoblastoid cells. Fusion and selection for independent hybrids were carried out as for hybridoma production (16). Subcloning was by limiting dilution.

Chromosome Analysis. The chromosome preparations were made either from peripheral blood cultures or lymphoblastoid cell lines. The technique used for obtaining prophase/prometaphase chromosomes from peripheral blood was by synchronization with methotrexate (17) or BrdUrd (18). Longer chromosomes were obtained from the lymphoblastoid cell lines by the use of ethidium bromide prior to harvesting (19). Giemsa banding (G banding) was obtained by the ASG technique (20) and reverse banding (R banding) was obtained by the method of Sehested (21).

From the somatic cell hybrids chromosome preparations were made by the ethidium bromide technique (19), Giemsa-banded by using the method of Gallimore and Richardson (22), destained overnight in 3:1 methanol/acetic acid, and finally stained by the Giemsa 11 technique (23) to identify human chromosomes unequivocally.

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Abbreviations: FACS, fluorescence-activated cell sorting; HBB, β-globin; CALC, calcitonin; PTH, parathyroid hormone; CAT, catalase; LDHA, lactate dehydrogenase A; AWT, aniridia–Wilms tumor; G banding, Giemsa banding; R banding, reverse banding.

Cell Surface Markers. Three monoclonal antibodies were used for fluorescence-activated cell sorting (FACS) analysis on the somatic cell hybrids. These antibodies recognize chromosome 11-encoded cell surface antigens in a species-specific manner. The antigens are expressed in several human tissues, including B-cell lines. F10.44.2 antibody (gift of John Fabre) and 163A5 antibody (gift of Peter Goodfellow) define two distinct antigens (13, 14, 24) encoded on the short arm of chromosome 11. TRA1.10 (gift of Peter Goodfellow) defines a long arm-encoded antigen (13, 24) and was used as the positive control antibody. The negative control antibody was AFP21.2, which recognizes human α-fetoprotein (16).

FACS Analysis. Cells were prepared for FACS analysis by using the procedure described by Andrews *et al.* (25) but with RPMI 1640 medium and 2% fetal calf serum as wash medium. Cells (1×10^6) were incubated with saturating amounts of first antibody, and fluorescein isothiocyanate-labeled sheep anti-mouse IgG, at a 1:40 dilution, was used as the second antibody. Cells were analyzed for the distribution of fluorescence intensity by using a FACS IV cell sorter. For each antibody, ≈10,000 cells were counted and data were obtained as a histogram of cell number plotted against relative fluorescence intensity.

Enzyme Analysis. LDHA isoenzyme analysis was by electrophoresis on "Cellogel" cellulose acetate membranes. Cell lysates, buffers, and staining reagents were prepared as described by Meera Khan (26). Relative LDHA activity was assessed visually.

DNA Extraction. DNA was extracted from lymphoblastoid cell lines and somatic cell hybrids as described in detail elsewhere (27).

Plasmids. The CAT cDNA-containing plasmid was isolated in this laboratory from a liver cDNA library by using a synthetic oligonucleotide as probe. The sequence of the insert has confirmed that it encodes CAT (unpublished data). The HBB cDNA-containing plasmid pPstβ has been described elsewhere (28). The CALC-cDNA containing plasmid PLT-B3 was a kind gift of S. Legon and I. MacIntyre and has been described (29). The PTH cDNA-containing plasmid p20.36 was a kind gift of J. Schmidtke, H. Mayer, and C. Bostock and has been reported elsewhere (30).

Gel Electrophoresis and Southern Analysis. DNA was digested with restriction endonucleases as recommended by the manufacturers and then electrophoresed in a 0.8% agarose gel. The DNA was then transferred to nitrocellulose as described by Southern (31).

Hybridization. Plasmids were labeled by using [α-^{32}P]TTP (800 Ci/mmol; 1 Ci = 37 GBq) by nick-translation (32) and hybridized to the filter at 68°C in a solution containing 0.75 M NaCl/0.075 M sodium citrate, 4× concentrated Denhardt's solution (Denhardt's solution: 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone), 10% dextran sulfate, 0.1% $NaDodSO_4$, 0.1% sodium pyrophosphate, and 100 μg of denatured salmon sperm DNA per ml. After hybridization overnight the filters were washed at 68°C in a solution containing 0.3 M NaCl/0.03 M sodium citrate, 0.1% $NaDodSO_4$, and 0.1% sodium pyrophosphate.

Densitometry. The bands on the autoradiogram were scanned and digitized by a 2048-element charge-coupled device array scanner designed and built in the Pattern Recognition Section of MRC Clinical and Population Cytogenetics Unit.

RESULTS

Clinical and Cytological Findings. Fig. 1 shows paired normal and deleted chromosomes 11 from the five patients. Band p13 is lightly stained by G banding but darkly stained by R banding. Chromosome nomenclature follows the 550-band diagram in ISCN 1981 (33).

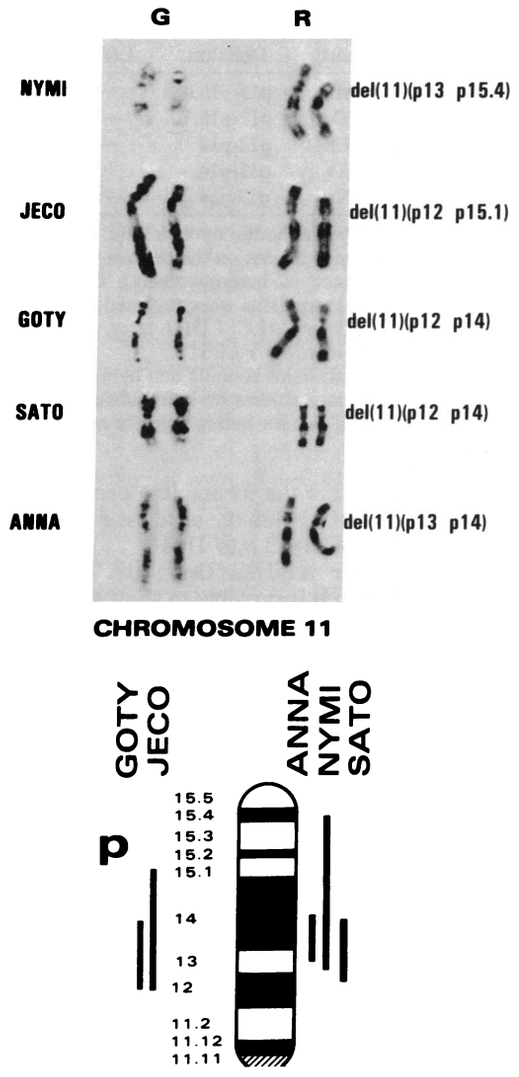

FIG. 1. Cytogenetic analysis of chromosome 11 deletions. (*Upper*) G and R banding of paired normal (left) and deleted (right) homologs from the five individuals. (*Lower*) Diagrammatic representation of the limits of the deletions.

NYMI, aged 12, has a deletion extending from the interface of 11p12/13 to p15.4. She has not had Wilms tumor but has bilateral aniridia and congenital cataracts and is severely mentally retarded. In addition, she has polydactyly of the feet, asymmetry of the cranial vault, and a loud pansystolic heart murmur. Interestingly, she has six instead of seven cervical vertebrae.

JECO, aged 24, has a deletion extending from 11p12 to 11p15.1. She also has not had Wilms tumor but has bilateral aniridia, is severely mentally retarded, has a systolic heart murmur, and is physically severely disabled.

GOTY, aged 12, has a deletion extending from 11p12 to 11p14. She has had a nephroblastoma removed, has bilateral aniridia, and is mildly mentally retarded.

SATO, aged 8, has very similar symptoms and deletion end points to GOTY. The deletion may be slightly less extensive, but the exact break points are difficult to determine.

The fifth patient, ANNA, is now 23 years old, has not had Wilms tumor but has aniridia and mild mental retardation. His twin brother, presumed monozygotic, also with aniridia, died 20 years ago after the removal of a nephroblastoma. This, like a similar reported case of discordant monozygotic

Table 1. Summary of marker analysis for the five chromosome 11 short arm deletions studied

Patient	Deletion	LDHA	163A.5	F10.44.2	CAT	PTH	HBB	CALC
NYMI	p13-p15.4	+*†	−*	−*	−*†	+*	+*	+*‡
JECO	p12-p15.1	−†	ND	ND	−†	ND	+‡	+‡
GOTY	p12-p14	−†	ND	ND	−†	ND	+‡	ND
SATO	p12-p14	+*†	−*	−*	−†	ND	ND	ND
ANNA	p13-p14	+*†	+*	+*	+*†	+*	+*	+*‡

The method(s) by which the assignments were made is indicated by superscripts. +, Gene present; −, gene absent on the deleted chromosome 11. ND, analysis not carried out or not informative (e.g., absence of heterozygosity). CAT, PTH, HBB, and CALC were studied by Southern blotting; polymorphisms were revealed by the restriction endonucleases *Pst* I for PTH, *Bam*HI for HBB, and *Taq* I for CALC; LDHA assays were by enzyme electrophoresis; 163A.5 and F10.44.2 expression was measured by FACS.

*Analysis of somatic cell hybrids.

†Dosage studies on lymphoblastoid cell line material.

‡Analysis for heterozygosity of polymorphic markers.

twins (34), demonstrates the incomplete penetrance of the Wilms mutation. ANNA bears the smallest deletion extending from the middle of 11p13 into 11p14.

Preliminary Marker Analysis Using the Established B-Lymphoblastoid Lines. It is possible to show that a polymorphic gene is outside a deletion region by demonstrating heterozygosity in the deleted individual. High molecular weight DNA from the five lymphoblastoid cell lines was analyzed by Southern blotting with the cDNA probes for HBB, PTH, and CALC. The restriction enzymes used were the ones known to reveal high-frequency polymorphisms. GOTY and JECO were found to be heterozygous for HBB (Table 1). NYMI, JECO, and ANNA were heterozygous for CALC (Table 1, Fig. 2 *Right*).

We also have evidence that the LDHA gene is deleted in JECO and GOTY but not in the other cell lines (Table 1).

DNA Marker Analysis in Somatic Cell Hybrids. Mouse–human B-cell somatic hybrids were produced by using NYMI, ANNA, and SATO.

Initial analysis was for the retention of the human LDHA subunit. LDHA-positive hybrids were subcloned for karyotypic stability and examined cytologically. For the NYX hybrids (human parent NYMI) the normal and deleted chromosomes 11 are easily distinguished. Subclones bearing each homolog were grown for preparation of high molecular weight DNA. Southern blot analysis was carried out by using the probes for HBB, CALC, PTH, and CAT. For HBB and CALC (Fig. 2 *Left*) and PTH (data not shown) the human-specific band(s) were present whenever either the deleted or the normal homolog was present. In contrast, the human CAT bands were absent when the deletion chromosome was present (Fig. 2 *Left*). A control hybrid with chromosome 11 as its sole human component confirms the assignment of the CAT structural gene to chromosome 11 (12).

In the ANX hybrids (human parent ANNA) it is difficult to distinguish the two homologs cytologically. Since ANNA was known to be heterozygous for a CALC restriction fragment length polymorphism (Fig. 2 *Right*), the presence of a different homolog in the two cytologically chosen hybrids was confirmed by the demonstration of a different CALC allele in each (Fig. 2 *Right*). Both of these hybrids, however, were found to possess the human-specific CAT bands (Fig. 2 *Right*), showing that the CAT gene lies outside the small deletion from the middle of the 11p13 into p14.

The SAX hybrids (human parent SATO) have not yet been studied by Southern analysis.

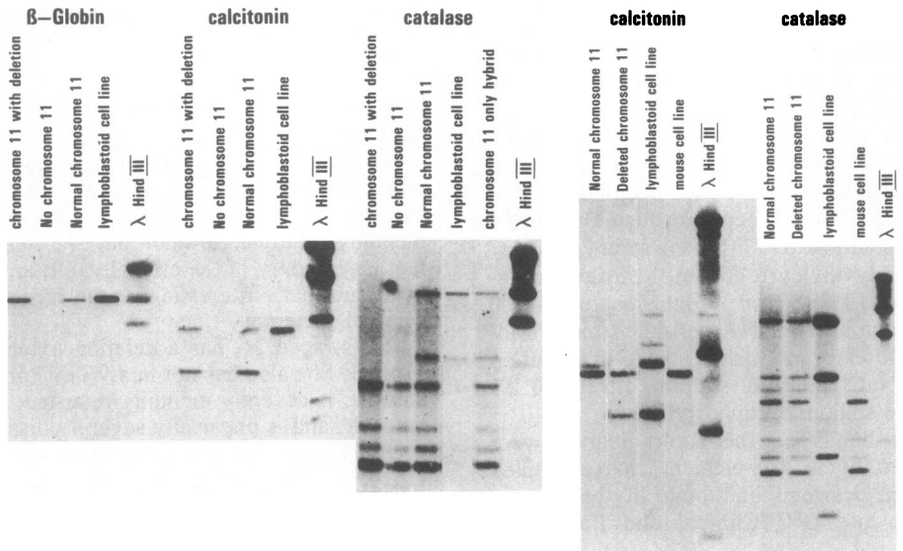

FIG. 2. Molecular analysis of the deletions in somatic cell hybrids. (*Left*) Southern blot analysis of DNA from the NYX somatic cell hybrids and the human parent cell line (NYMI). Hybrid cell and lymphoblastoid cell DNA was digested with *Taq* I for the HBB analysis and with *Pst* I for the other probes. Five micrograms of DNA was loaded in each track, except that 20 μg was used for the CAT analysis of the somatic cell hybrids (which included a chromosome 11 only hybrid). (*Right*) Southern blot analysis of DNA from the ANX somatic cell hybrids and the human parent cell line (ANNA). DNA from the cell lines was digested with *Taq* I for CALC or *Pst* I for CAT analysis. Amounts of DNA loaded in each track were as in *Left*.

Analysis of CAT Gene Deletions by Dosage. Having demonstrated CAT gene status of deletions in somatic cell hybrids, we examined CAT gene dosage directly in DNA prepared from deletion cell lines and controls. DNA was digested with a restriction enzyme chosen to produce clearly separated, nonpolymorphic bands on simultaneous hybridization with probes for CAT and HBB. HBB was known not to be deleted in four of the patients (Table 1) and could therefore be used as an internal standard. Fig. 3 shows Southern blots for the largest HBB and CAT fragments and also shows the densitometric tracing and peak ratios obtained for pairwise combinations of patients and controls.

We have carried out this type of analysis several times and, though there is some variability, there is no doubt from the HBB:CAT ratios that NYMI, SATO, GOTY, and JECO all lack one copy of the CAT gene, whereas ANNA does not.

FACS Analysis for the Expression of Cell Surface Antigens. FACS analysis was carried out for the expression of two short arm-encoded antigens and of one long arm one. Initially, we studied the ANX and NYX hybrids bearing either the deleted or the undeleted chromosomes 11. Fig. 4 shows that NYX5.6, known to carry NYMI's undeleted chromosome 11, expresses both of the short arm markers. However, NYX3.1 expresses neither antigen, although the deleted chromosome 11 was present in 90% of the cells at the time of analysis. ANX8.1 bears the deleted chromosome 11 from ANNA. Both of the antigens are expressed in ≈50% of the cells analyzed. The reason for this heterogeneity of antigen expression is the chromosomal instability of this clone. We have confirmed cytologically that cells in the positive peak contain the deleted chromosome 11. Thus, both of the short arm-encoded cell surface antigens are deleted in NYMI's large deletion but not in ANNA's small one.

FACS analysis was carried out on early, unstable LDHA-positive SAX hybrids. Of the first two lines tested, one possessed all three chromosome 11-encoded antigens; the other, SAX3, was positive only for the long arm marker (data not shown). Cells selected for expression of the long arm marker still lacked both short arm markers (Fig. 4). Chromosome analysis confirmed the presence of the deleted chromosome 11 homolog in these cells. The genes for both short arm antigens are deleted in SATO.

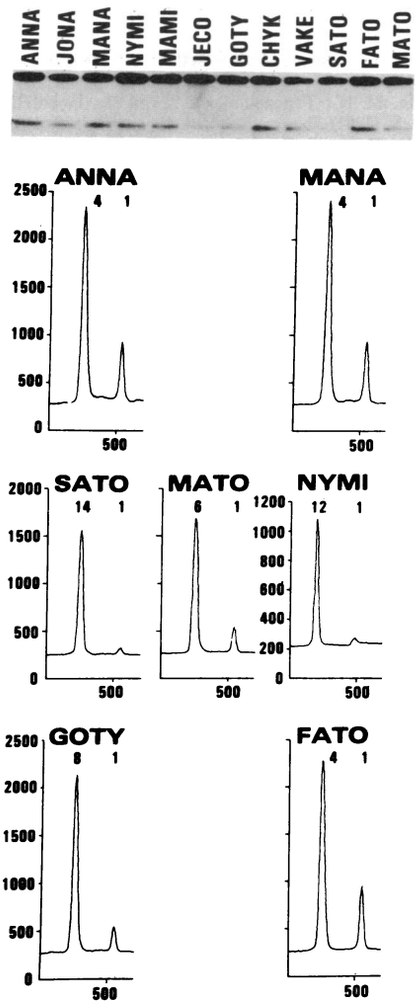

FIG. 3. Relative dosage analysis for HBB and CAT genes. Five micrograms of lymphoblastoid cell line DNA from the individuals listed was digested with *Taq* I. Following electrophoresis and transfer, the DNA was hybridized with a 5:1 mixture (on the basis of radioactivity) of CAT and HBB probes. (*Upper*) Autoradiograph showing the largest HBB (upper bands) and CAT (lower bands) gene fragments. FATO and MATO are cell lines from the father and mother of SATO; JONA and MANA are cell lines from the father and mother of ANNA; MAMI is a cell line from the mother of NYMI. CHYK and VAKE are unrelated control cell lines. This is a 7-day autoradiographic exposure. (*Lower*) Densitometric tracings of autoradiographic tracks shown in *Upper*. Note: The NYMI panel is a tracing of a 3-day autoradiographic exposure of the same gel.

DISCUSSION

By a combination of hybrid cell and dosage analysis we have shown, at the DNA level, that one copy of the CAT gene is missing in four of five individuals with visible deletions. The demonstration of deletion by gene dosage rules out the possibility of CAT being translocated to another chromosome. ANNA is not deleted for CAT, indicating that the CAT gene may map up to several thousand kilobases away from the Wilms and aniridia loci. It is the only line in which the whole of band p13 is not deleted: the proximal half remains. Considered together with the repeated assignment to the p13 region of the gene for CAT (for reviews, see refs. 4 and 5), our data suggest that CAT maps to the centromere-proximal part of band p13. Such an assignment is consistent with the description by Turleau *et al.* (35) of a patient with Wilms tumor and reduced CAT enzyme levels but no aniridia and with the proximal part of 11p13 deleted. However, it contradicts the findings of Narahara *et al.* (36), who placed CAT distally to the aniridia and Wilms tumor genes.

Table 1 summarizes our findings for the five chromosome 11 deletions studied. The data are consistent with the original cytogenetic analysis.

The retention of the gene for HBB in a series of overlapping deletions (NYMI, ANNA, GOTY, and JECO) extending from the proximal end of p12 to p15.4 places HBB outside these two end points. This is consistent with assignments of HBB to the p15 terminal region (37–39).

Similarly, we conclude that the CALC gene must lie outside the p12-p15.4 region of chromosome 11. This is difficult to reconcile with the assignment of this gene to p14 by *in situ* hybridization (40) but could be consistent with its localization to 11p14-qter (41).

The gene encoding PTH is excluded by our findings from the region between the proximal end of p13 and p15.4. Since PTH has been found to be closely linked to HBB (42), the likely localization is at a position distal to p15.4.

The reduction in the enzyme activity levels of the LDHA isoenzyme relative to LDHB, in GOTY and JECO, is consistent with LDHA assignment to 11p12 (43) and our cytogenetic finding of the most centromere-proximal break points in these patients (Fig. 1).

The genes for the two short arm-assigned cell surface antigens F10.44.2 and 163A.5 are located outside the ANNA deletion (middle of p13-p14) but within both the SATO

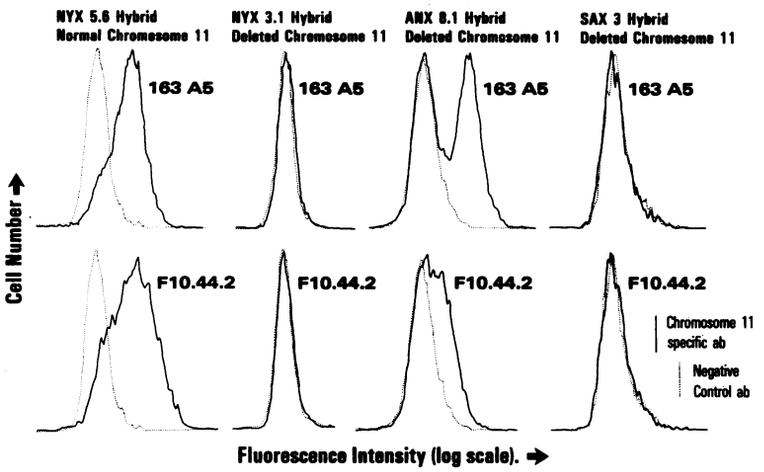

FIG. 4. FACS analysis for the expression of two cell surface markers in the somatic cell hybrids. In each case negative control calibration was carried out with a monoclonal antibody directed to α-fetoprotein. ab, Antibody.

deletion (distal end of p12-p14) and the NYMI deletion (p13-p15.4) regions. Therefore, from the combined data we conclude that the two cell surface antigen loci lie between the proximal end of p13 and the middle of the p14 band. Because the exact breakpoints are impossible to define cytologically, we cannot conclude whether both of the loci are on the proximal or distal side of the ANNA deletion.

The localization of genes encoding two distinct cell surface antigens to just outside the region extending from the middle of p13 to some way into p14 is fortunate. These are selectable markers that will be useful for isolating and maintaining somatic cell hybrids bearing other chromosome 11 deletions (44) and translocations. They can also be used for isolating fragments of chromosome 11 introduced into rodent cells by chromosome-mediated transfection. This will aid the construction of DNA libraries specific for the region of interest.

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