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Authors

Lambert, C
Schultz, RA
Smith, M
et al.

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Functional complementation of ataxia–telangiectasia group D (AT-D) cells by microcell-mediated chromosome transfer and mapping of the AT-D locus to the region 11q22–23

(gene transfer/hereditary diseases/ionizing radiation/cell cycle/DNA synthesis)

CLARE LAMBERT*, ROGER A. SCHULTZ†, MOYRA SMITH‡, CARYN WAGNER-MCPHERSON‡, LISA D. MCDANIEL†, TIM DONLON*, ERIC J. STANBRIDGE§, AND ERROL C. FRIEDBERG*¶

*Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305; †Division of Human Genetics, University of Maryland, Baltimore, MD 21201; and Departments of ‡Pediatrics and §Microbiology and Molecular Genetics, University of California, Irvine, CA 92717

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ABSTRACT **The hereditary human disease ataxia–telangiectasia (AT) is characterized by phenotypic complexity at the cellular level. We show that multiple mutant phenotypes of immortalized AT cells from genetic complementation group D (AT-D) are corrected after the introduction of a single human chromosome from a human–mouse hybrid line by microcell-mediated chromosome transfer. This chromosome is cytogenetically abnormal. It consists primarily of human chromosome 18, but it carries translocated material from the region 11q22–23, where one or more AT genes have been previously mapped by linkage analysis. A cytogenetically normal human chromosome 18 does not complement AT-D cells after microcell-mediated transfer, whereas a normal human chromosome 11 does. We conclude that the AT-D gene is located on chromosome 11q22–23.**

The hereditary disease ataxia–telangiectasia (AT) is characterized by a pleomorphic clinical presentation that includes cerebellar ataxia, permanent dilatation of small blood vessels (telangiectasia), and immunodeficiency (1, 2). Approximately 35% of afflicted individuals develop malignancies, the majority of which are of the lymphoid system, including Hodgkin disease, non-Hodgkin lymphomas, and lymphocytic leukemia (3).

At the cellular level the phenotype of AT cells is also complex. Cells are abnormally sensitive to killing by ionizing radiation and by x-ray-mimetic chemicals (4). In addition, inhibition of DNA synthesis observed in normal cells exposed to ionizing radiation is replaced by a radioresistant DNA synthesis mode and AT cells manifest reduced levels of DNA synthesis in the unirradiated state (5). AT cells show a block in the G_2/M phase of the cell cycle after exposure to ionizing radiation (6, 7), and chromosomal instability is very characteristic (8, 9).

The disease has an autosomal recessive mode of inheritance (3). Complementation of radioresistant DNA synthesis in heterodikaryons generated by fusion of fibroblast lines from different AT individuals suggests the existence of at least four genetic complementation groups for the classical form of the disease—designated AT-A, AT-C, AT-D, and AT-E (10). Two variant complementation groups have also been defined (10). The AT-A gene has been mapped to the region 11q23 by linkage analysis of families assigned to genetic complementation group A (11). In addition, the AT-C locus has been mapped to the region 11q22–23 by linkage analysis on a single Jewish–Moroccan family (12). Linkage studies on mixtures of AT families from genetic complementation groups A, C, and E, as well as multiple unassigned families, do not provide evidence for the location of any AT genes outside the region 11q22–23 (11, 13, 14). However, the numbers of families from group E are too small to provide unequivocal genetic mapping. Furthermore, none of these studies included families known to belong to complementation group D. Hence, at the present time it is not clear whether normal cells carry a single AT gene or multiple apparently linked genes.

The marked sensitivity of AT cells to ionizing radiation and x-ray-mimetic chemicals offers a selectable phenotype for functional cloning of wild-type AT genes. However, transfection of total human genomic DNA into human cells presents special problems associated with the limited efficiency of stable integration and gene expression (15–17). Hence, the isolation of stable radiation-resistant AT cell lines after transfection with total human genomic DNA has been unsuccessful (18) and no AT genes or gene products have yet been isolated.

As a prelude to developing alternative strategies for gene cloning, we have used the technique of microcell-mediated chromosome transfer (19) to introduce single human chromosomes into AT cells. We show here that the transfer of a single rearranged human chromosome to AT group D (AT-D) cells results in correction of multiple mutant phenotypes. The complementing chromosome consists largely of human chromosome 18 but contains translocated material from the region 11q22–23. Microcell-mediated transfer of a cytogenetically normal chromosome 18 does not complement the phenotype of AT-D cells; however, transfer of chromosome 11 does. The isolation of a single human chromosome containing a limited region of chromosome 11 offers the potential for molecular cloning of the AT-D gene.

MATERIALS AND METHODS

Cell Lines and Growth Conditions. Two independently derived series of pooled mouse–human hybrid cells were used as the source of donor human chromosomes. One series of hybrids (LD) was constructed with normal human diploid cells containing *neo*-tagged chromosomes obtained from T. W. Glover (University of Michigan). The other series (VA) was derived from a simian virus 40 (SV40) immortalized human line (VA-neo) described previously (20). In both cases, the hypoxanthine phosphoribosyltransferase-negative mouse line A9 was used to generate hybrids. The monochromosomal hybrid lines MCH556.1 and MCH912.1 carry cytogenetically normal human chromosomes 11 and 18, respec-

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Abbreviations: AT, ataxia–telangiectasia; SV40, simian virus 40; FBS, fetal bovine serum.

¶To whom reprint requests should be sent at present address: Department of Pathology, The University of Texas Southwestern Medical Center at Dallas, Dallas, TX 75235.

tively (E.J.S., unpublished observations). Hybrid lines were grown in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), nonessential amino acids, and the antibiotic geneticin (G418) (400 μg/ml) to maintain selection for *neo*-tagged human chromosomes.

The SV40-transformed human cell line VA-neo was used as the wild-type control. The SV40-transformed AT cell line AT5BI (genetic complementation group D) was obtained from the Genetic Mutant Cell Repository (cell line GM5849). All cell lines were maintained in EMEM plus nonessential amino acids with 10% FBS, except for AT5BI cells, which required 15% FBS. AT lines carrying a *neo*-tagged human chromosome were grown as described above with the addition of G418 at 250 μg/ml.

Plasmids and DNA Probes. Probes D18S3, D18S5, and D18S14 were supplied by Uta Francke (Department of Genetics, Stanford University). Probe pWI-25 was a gift of Hunt Willard (Department of Genetics, Stanford University). BCL2 was obtained from Michael Cleary (Department of Pathology, Stanford University), and probes D18S6, YES1, and MBP were purchased from the American Type Culture Collection. A chromosome 18-specific phage λ library was generously supplied by Dan Pinkel (Lawrence Livermore National Laboratory, Livermore, CA). Human-specific *Alu* and LINE1 PCR probes from the human–mouse hybrid cell line X31sub7 were constructed as described (21), except that the probes were labeled with biotin by including biotin-dUTP during the PCR and by nick-translation after the reaction. Probes spanning the region 11q14–11qter are identified in Table 1.

Characterization of the Complementing Chromosome. Single human chromosomes in the hybrids X31sub7, MCH556.1, and MCH912.1 were identified by fluorescence *in situ* hybridization (25), using total human genomic DNA as a probe, and were analyzed cytogenetically by the GTW banding technique (26). The presence of chromosome 18 in the hybrid X31sub7 was confirmed by fluorescence *in situ* hybridization using a chromosome 18-specific library. This was confirmed by fluorescence *in situ* hybridization to metaphase spreads of normal human female fibroblasts using *Alu* and LINE1 PCR probes as described (21), and by Southern hybridization with ^{32}P-labeled chromosome 18-specific probes. The presence of chromosome 11 material was demonstrated by fluorescence *in situ* hybridization using single copy chromosome 11-specific probes, and by Southern hybridization or PCR using chromosome 11-specific probes or primers. This was confirmed by fluorescence *in situ* hybridization using *Alu* and LINE1 PCR probes as described (21).

Functional Complementation of AT Cells by Microcell-Mediated Chromosome Transfer. The *neo*-tagged human chromosomes were transferred to recipient AT5BI cells by microcell-mediated chromosome transfer as described (19). Microcells were fused to ≈5 × 10^5 recipient AT cells seeded in 100-mm dishes. Single G418-resistant colonies were clonally expanded in 24-well plates and each clone was qualitatively screened for streptonigrin resistance by plating ≈500 cells in EMEM with 15% FBS, or in EMEM with 15% FBS plus streptonigrin (0.05 ng/ml). Surviving AT clones were rescreened and then quantitatively characterized in terms of streptonigrin resistance, γ-ray resistance, radioresistant DNA synthesis, and cell cycle distribution.

Table 1. Analysis of chromosome 11q markers in the human–mouse hybrid cell line X31sub7

Locus/probe	Map position	Detection method	Hybridization signal
TYR/pMEL34	11q14–21	So	−
D11S35/phi2-22	11q21–22	IS, P	−
PGR	11q22–23	So	−
D11S384/CJ52-193	11q22–23	IS, P	−
D11S424/CJ52-77	11q22–23	IS, So, P	−
D11S132/ CRIL424	11q22–23	So, P	+
DRD2(5'9HI)	11q22–23	IS	+
DRD2(3'9A5)	11q22–23	IS, P	+
NCAM	11q23	IS	+
PBGD/6C1	11q23.2–qter	IS, So	−

Probe assignments are provided in refs. 22–24. So, Southern hybridization; IS, *in situ* hybridization; P, PCR analysis.

Cell Survival. Survival of cells after chronic treatment with streptonigrin was quantitated by plating cells in 100-mm dishes at various concentrations of streptonigrin for 24 hr. The treatment was repeated and after a further 24 hr the medium was replaced with EMEM plus 10% (VA-neo cells) or 15% (AT5BI cells) FBS and the cells were incubated for a further 10–14 days. Cells were fixed and stained as described (20) and sensitivity to streptonigrin was quantitated by counting colonies. Survival after exposure to γ-rays was quantitated by exposure of cells at 5 × 10^5 cells per ml to a ^{137}Co source at a dose rate of 1.7 krad/min (1 rad = 0.01 Gy). Cells were plated in 100-mm dishes at appropriate dilutions and counted after 10–14 days.

Measurement of Radioresistant DNA Synthesis. Cells in exponential growth were either mock irradiated or γ-irradiated at a dose of 450 rad in double-well tissue culture slides at a density of 3.6 × 10^5 cells per well. Cells were irradiated in phosphate-buffered saline and then incubated in fresh growth medium for 30 min. The cells were pulse labeled with 0.25 μCi of [^{3}H]thymidine per ml (specific activity, 46 Ci/mmol; 1 Ci = 37 GBq) in growth medium for 60 min, rinsed once in 0.9 M NaCl, and prepared for autoradiography as described (10).

Cell Cycle Analysis. Exponentially growing cells were either mock irradiated or γ-irradiated at a dose of 400 rad in serum-free medium at a concentration of 10^5 cells per ml. After incubation in growth medium for 12 hr, cells were fixed in 70% ethanol for 1 hr, stained with chromomycin as described (27), and analyzed in an Epics model 753 fluorescence-activated cell sorter.

Rescue of the Complementing Chromosome. The complementing chromosome was rescued from the AT5BI cell line by fusion with mouse A9 cells. A mixture of equal numbers of the two cell lines was seeded in T150 flasks and after attachment the medium was removed and the cells were fused by addition of 50% polyethylene glycol for 2 min. Selection for whole cell hybrids was carried out in the presence of G418 (400 μg/ml) and ouabain (2 μM). The single *neo*-tagged human chromosome was transferred to mouse A9 cells by microcell transfer, thereby generating a new series of monochromosomal human–mouse hybrid lines. The chromosome was then retransferred to AT5BI cells to verify phenotypic complementation.

RESULTS

Phenotypic Complementation of AT-D Cells. Consistent with previous observations, we demonstrated that AT cells from the immortalized fibroblast line AT5BI [genetic complementation group D (10)] are hypersensitive to killing after exposure to γ-radiation or to the radiomimetic chemical streptonigrin. Plating cells and growing them in the presence of low doses of streptonigrin for an extended time resulted in optimal differential sensitivity of AT and wild-type cells (Fig. 1). We therefore used chronic treatment with low doses of streptonigrin to screen AT cells for phenotypic complementation.

Different pools of human–mouse hybrid lines carrying random *neo*-tagged human chromosomes were used for chromosome transfer to AT5BI cells. Recipient cells were se-

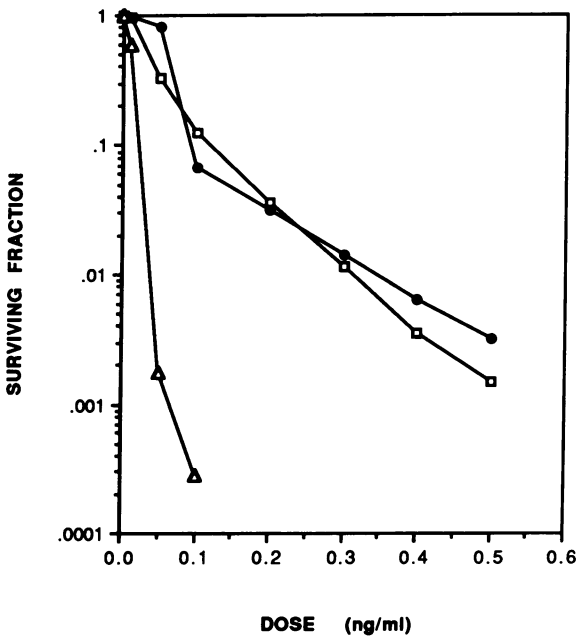

FIG. 1. Complementation of the sensitivity of AT5BI cells to streptonigrin. □, Streptonigrin-resistant cell line (VA-neo); ●, AT5BI cells carrying the complementing human chromosome; △, AT5BI cells.

lected for resistance to the antibiotic geneticin (G418) and were then screened for increased resistance to killing by streptonigrin. Transfer of human chromosomes from most hybrid pools had no effect on the streptonigrin sensitivity of AT5BI cells. After microcell-mediated transfer from a pool designated X31 (from the LD series of hybrids), G418-resistant ($G418^R$) colonies showed enhanced resistance to streptonigrin at levels indistinguishable from the streptonigrin-resistant line VA-neo (Fig. 1).

A clonal population of human–mouse hybrids (designated X31sub7) containing a single human chromosome was isolated from the X31 pool. The single human chromosome was readily distinguished from mouse chromosomes by G-11 staining and by *in situ* hybridization to metaphase spreads using total human genomic DNA as a probe (Fig. 2). Transfer of G418 resistance to AT5BI cells from this hybrid was accompanied by resistance to streptonigrin in all cases. These cells also showed enhanced resistance to γ-radiation and to bleomycin.

To confirm that complementation of AT5BI cells was specifically associated with the presence of the single *neo*-tagged human chromosome, we rescued this chromosome by fusing the complemented AT5BI cells to mouse A9 cells. The resulting human–mouse hybrids were subcultured in the presence of G418 to allow segregation of untagged human chromosomes. Subsequent chromosome transfer to mouse A9 cells and selection for G418 resistance established a new series of monochromosomal hybrids. One of these (designated MATA9-9) was used to transfer G418 resistance to AT5BI cells, once again resulting in complementation of sensitivity to streptonigrin at levels similar to that observed previously.

Genomic DNA from two complemented AT5BI clones was examined for the presence of mouse DNA sequences by dot blot hybridization with radiolabeled mouse genomic DNA. No rodent sequences were detected. Based on the sensitivity of this hybridization assay we conclude that the complemented AT cells contain <0.001% mouse sequences.

The complementing chromosome was relatively stable in AT5BI cells in the absence of selection. When three independent clones were subcultured over a period of 12 weeks

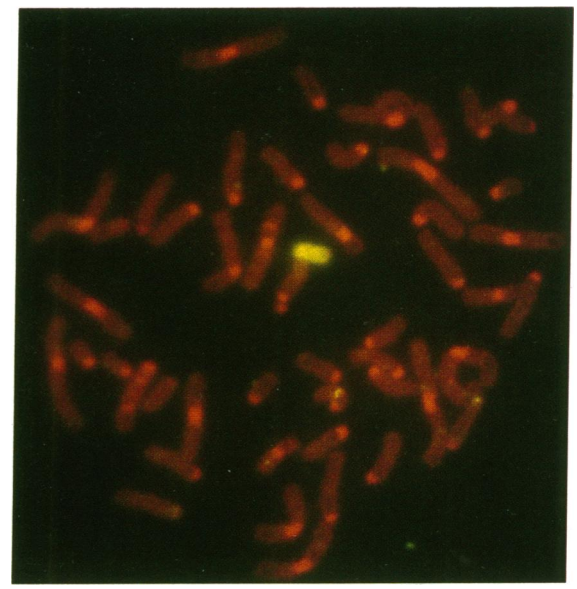

FIG. 2. Fluorescence *in situ* hybridization of metaphase spreads of the monochromosomal human–mouse hybrid cell line X31sub7 after hybridization with total human genomic DNA. The single human chromosome is identified by its yellow fluorescence in contrast to the red counterstained mouse chromosomes.

without G418, 50%, 84%, and 81% of the cells, respectively, retained both the $G418^R$ and streptonigrin-resistant determinants. In the majority of cases in which resistance to streptonigrin was lost the $G418^R$ determinant cosegregated. No streptonigrin-resistant G418-sensitive segregants were detected. In some clones of X31sub7 maintained in culture for many months the human chromosome underwent duplication.

Complementation of Other Cellular Phenotypes. In view of the complex cellular phenotype of AT we investigated whether transfer of the single human chromosome was associated with complementation of other phenotypes in AT5BI cells. Consistent with the results of previous studies (5), we observed that unirradiated AT5BI cells carried out reduced levels of DNA synthesis relative to wild-type cells (Fig. 3). This phenotype was fully complemented in AT5BI cells carrying the transferred human chromosome. After exposure to γ-radiation wild-type cells showed inhibition of DNA synthesis. In contrast, the extent of DNA synthesis in irradiated AT5BI cells remained similar to that in unirradiated cells. Cells carrying the transferred human chromosome regained the radiosensitive DNA synthesis phenotype of wild-type cells.

SV40-transformed AT cells accumulate at the G_2/M phase of the cell cycle after exposure to γ-radiation or to other DNA-damaging agents (6, 7). Flow cytometry of fluorescent γ-irradiated cells showed the wild-type population (VA-neo) to be distributed bimodally as 2n and 4n cells, reflecting the G_1/S and G_2/M stages of the cell cycle, respectively (Fig. 4). In contrast, AT5BI cells not carrying extra chromosomes (data not shown) or those carrying a transferred noncomplementing human chromosome were mainly distributed as a single 4n peak, reflecting an accumulation at the G_2/M phase of the cycle. After microcell-mediated transfer of the single human chromosome to AT5BI cells, a wild-type pattern was restored.

Characterization of the Complementing Chromosome. The hybrid line X31sub7 carrying the complementing human chromosome was characterized cytogenetically and by DNA hybridization. Examination of cells by the GTW banding technique showed the presence of an abnormal human chromosome 18. Fluorescence *in situ* hybridization of the hybrid

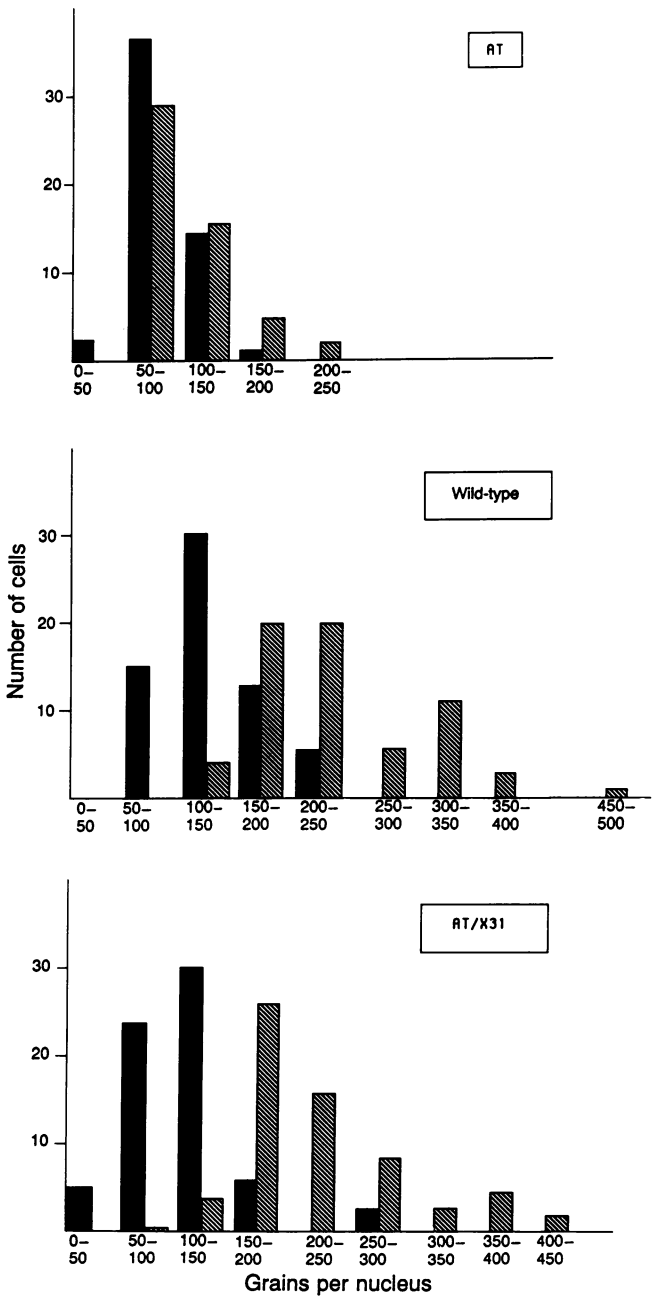

FIG. 3. Complementation of defective DNA synthesis in unirradiated and γ-irradiated AT cells after transfer of a single human chromosome. Semiconservative DNA synthesis in AT5BI (*Top*), wild-type (*Middle*), and complemented AT5BI (*Bottom*; AT/X31) cells was determined by autoradiography. Hatched bars represent the amount of DNA synthesis in unirradiated cells. Solid bars represent DNA synthesis in cells exposed to γ-radiation.

with a chromosome 18-specific library painted the entire human chromosome. The presence of chromosome 18 was further confirmed by Southern hybridization with DNA probes specific for both the p and q arms and for the centromere.

While this study was in progress it was reported that the sensitivity of AT-D cells to x-irradiation is complemented by microcell-mediated transfer of human chromosome 11 (28). In view of these results and of the abnormal morphology of the complementing chromosome identified in our studies, we carried out independent transfers of cytogenetically normal human chromosome 11 and 18 to AT-D cells. The former chromosome fully complemented sensitivity to streptonigrin

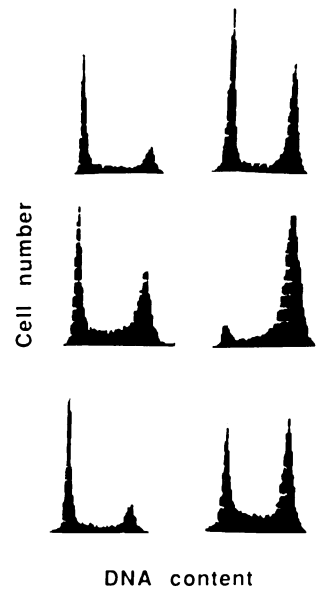

FIG. 4. Complementation of the cell cycle defect in γ-irradiated AT cells carrying a transferred human chromosome. Cell cycle analysis of wild-type GM5849 cells (*Top*), AT5BI cells from genetic complementation group D carrying a noncomplementing transferred human chromosome (*Middle*), or AT5BI cells carrying the complementing human chromosome (*Bottom*), was carried out by flow cytometry of unirradiated (*Left*) or γ-irradiated (*Right*) cells stained with chromomycin. The peak on the left of each profile reflects cells in G_1 phase of the cell cycle, the peak on the right reflects cells in the G_2/M phases, and the area between the peaks reflects S-phase cells.

and to γ-rays. Comparable results were obtained after the transfer of a rearranged human chromosome previously shown to carry just the q arm of chromosome 11 (29). Transfer of human chromosome 18 did not result in correction of these phenotypes. When the complementing chromosome was reisolated from complemented AT-D cells, it once again complemented streptonigrin and γ-ray sensitivity.

These results confirm and extend the observation that the AT-D gene is located on human chromosome 11 and suggest that the complementing chromosome in the hybrid X31sub7 contains chromosome 11 material, which is not recognizable by conventional cytogenetics. Fluorescence *in situ* hybridization of normal human cells with human-specific *Alu* and LINE1 PCR probes derived from the hybrid X31sub7 yielded signals in chromosomes 18 and 11 (data not shown). In addition, discrete signals were detected by fluorescence *in situ* hybridization of the hybrid using DNA probes mapped to the region 11q22–23 (Table 1 and Fig. 5). This was confirmed by Southern hybridization and by PCR analysis (Table 1). Hybridization reactions with 11q probes located both proximal and distal to the region 11q22–23 were negative (Table 1). The intensity of the signals obtained by *in situ* hybridization (Fig. 5) suggests that at least some of the translocated region of chromosome 11 might be amplified.

DISCUSSION

The increased sensitivity of AT cells to killing by selective DNA-damaging agents invites a molecular approach to the study of this disease, based on the cloning of AT genes by functional complementation. However, attempts to demonstrate stable complementation after transfection of immortalized AT cell lines with total human genomic DNA have not been successful (18). These failures are not unique to AT cell lines. Similar difficulties have been reported with cell lines from other hereditary human diseases with complementable phenotypes (e.g., xeroderma pigmentosum) and reflect a

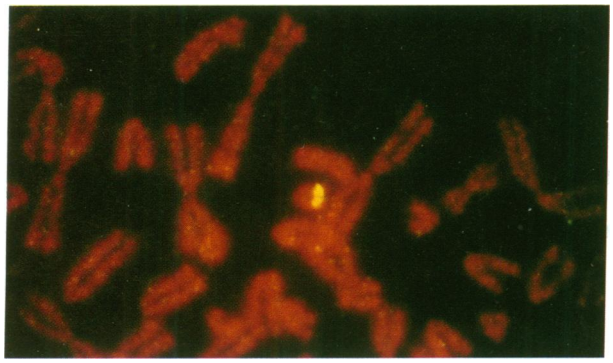

FIG. 5. Fluorescence *in situ* hybridization analysis of the hybrid X31sub7 with a mixture of the DNA probes D11S35, DRD2 (5'9H1), and NCAM, all of which have been mapped to the region 11q22–23 (see Table 1). Discrete fluorescence signals are present in both chromatids of the single human chromosome. The intensity of the hybridization signal shown here was similar when individual DNA probes were used, suggesting that this region of the rearranged chromosome might be amplified.

more general problem associated with the limited efficiency of stable integration in immortalized human fibroblasts transfected with total human genomic DNA (15–18).

We and others have utilized the technique of microcell-mediated chromosome transfer to investigate phenotypic complementation of cell lines from hereditary human diseases with defective cellular responses to DNA damage. Human chromosomes tagged with dominant selectable markers can be selected in recipient cells of interest and maintained in monochromosomal human–mouse hybrids (19). Different single human chromosomes from repair-proficient cells complement both increased sensitivity to killing by ultraviolet radiation and defective excision repair of DNA in xeroderma pigmentosum cells from genetic complementation groups A (29–31), F (32), and D (R.A.S. and E.C.F., unpublished observations). This experimental system thus affords an alternative approach to exploring the molecular basis of diseases such as xeroderma pigmentosum and AT.

The observation that multiple mutant phenotypes of AT5BI cells were complemented after transfer of a single human chromosome supports the notion that they derive from a single genetic defect. Linkage studies on families from AT group A individuals have mapped the AT-A locus to the region 11q23 (11). Additional linkage studies with families from genetic complementation groups A, C, and E, as well as uncharacterized AT families, are consistent with the notion that all AT genes map to this region (11–14). Our results using microcell-mediated chromosome transfer indicate that the AT-D locus is also located in the region 11q22–23 and hence support the contention that many, if not all, AT loci map to this region.

Assuming that phenotypic complementation of radioresistant DNA synthesis in heterodikaryons from different AT individuals reflects the existence of different genetic loci, normal cells must contain multiple closely linked AT genes. Alternatively, functional complementation in AT heterodikaryons may be intragenic, in which case a single AT gene may be represented in the population by multiple mutant alleles. The availability of DNA probes in the region 11q22–23 offers the potential for molecular cloning of the AT-D gene by identifying selected clones from recombinant chromosome 11-specific libraries and screening these for functional complementation of AT cells. Cloning and sequencing of the gene will facilitate the examination of genes from other genetic complementation groups to definitively determine whether or not multiple alleles exist.

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