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# Expression Cloning of Multiple Human cDNAs That Complement the Phenotypic Defects of Ataxia-Telangiectasia Group D Fibroblasts

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## Summary

**Ataxia-telangiectasia (A-T) is an inherited human disease of unknown etiology associated with neurologic degeneration, immune dysfunction, cancer risk, and genetic instability. A-T cells are sensitive to ionizing radiation and radiomimetic drugs, offering the possibility of cloning A-T genes by phenotypic complementation. We have used this sensitivity to isolate the first human cDNAs reported to complement A-T cells in culture. Complementation group D A-T fibroblasts were transfected with an episomal vector-based human cDNA library, ~610,000 resultant transformants were treated with the radiomimetic drug streptonigrin-resistent, and nine unrelated cDNAs were recovered from 29 surviving streptonigrin-resistant clones. Five cDNAs were mapped, but none localized to 11q23, the site of A-T complementation group A and C loci. Four of the mapped cDNAs conferred mutagen resistance to A-T D fibroblasts on secondary transfection. One cDNA was identified as a fragment of *dek*, a gene involved in acute myeloid leukemia. The *dek* cDNA fragment and pCAT4.5, a 4.5-kb cDNA that mapped to 17p11, independently complemented three different phenotypic abnormalities of A-T D fibroblasts (mutagen sensitivity, hyperrecombination, and radio-resistant DNA synthesis). The pCAT4.5 cDNA did not complement the mutagen sensitivity of an A-T group C fibroblast line, suggesting that it represents a candidate disease gene for group D A-T. Our results indicate that phenotypic complementation alone is insufficient evidence to prove that a candidate cDNA is an A-T disease gene. The complementing cDNAs may represent previously uncharacterized genes that function in the same pathway as does the A-T gene product(s) in the regulation of cellular responses to DNA damage.**

## Introduction

Ataxia-telangiectasia (A-T) is an autosomal recessive disease characterized by progressive cerebellar ataxia, progeric changes of the skin, immune defects, endocrine disorders, gonadal abnormalities, and a high incidence of cancer (Hecht and Hecht 1990; Sedgwick and Boder 1991). Heterozygote carriers may also be at increased risk for cancer, particularly breast carcinoma (Swift et al. 1987). Patients with the classic form of A-T have been divided into four complementation groups (A, C, D, and E) on the basis of heterodikaryon complementation studies (reviewed in Cohen and Levy 1989). A-T cells exhibit, in vivo and in vitro, abnormalities consistent with a defect involving DNA metabolism and/or maintenance of genomic integrity: e.g., elevated frequencies of chromosome aberrations (Cohen and Levy 1989) and intrachromosomal recombination (Meyn 1993), aberrant immune gene rearrangements (Cohen and Levy 1989), sensitivity to the killing effects of ionizing radiation (Cohen and Levy 1989), and defective damage-responsive cell-cycle checkpoints (Cohen and Levy 1989; Kastan et al. 1992). As a result, A-T represents an important model system for the study of cellular responses to induced and spontaneous DNA damage as well as neurologic degeneration, immunodeficiency, and cancer.

Despite extensive investigation, no A-T gene has been identified to date, and the site of action of the A-T gene product(s) is unknown. The sensitivity of A-T cells

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to the killing effects of ionizing radiation and radiomimetic chemicals offers the possibility of cloning A-T genes, as well as other genes involved in the same pathway, by complementation of the A-T phenotype in culture. However, efforts to isolate these genes by complementation with genomic human DNA have been impeded by low transfection frequencies, a tendency to integrate relatively small (<50 kb) segments of DNA, instability of integrated DNA, and difficulties in recovering transfected genes from complemented cells (Hoeijmakers et al. 1987; Loher et al. 1988; Mayne et al. 1988).

To circumvent these difficulties, we created a human cDNA expression library in pRep5, an Epstein-Barr virus (EBV)–based cDNA expression vector, for use in phenotypic complementation experiments. This shuttle vector is maintained episomally in human cells and bacteria (Groger et al. 1989). As a result, cDNAs contained in the vector can be rescued from complemented cells by direct transfection of nuclear DNA into *Escherichia coli,* thus enhancing transfection efficiency and ease of recovery (Strathdee et al. 1992). We transfected the cDNA library into an SV40-transformed group D A-T fibroblast line and then selected for complementation of mutagen sensitivity. We recovered nine unrelated cDNAs from transfected clones that exhibited correction of one or more aspects of the mutant phenotype. Five unrelated cDNAs, including a fragment of the putative oncogene *dek,* corrected one or more aspects of the A-T phenotype when transfected independently into A-T D fibroblasts.

## Material and Methods

### *cDNA Library Construction*

A cDNA expression vector library was constructed using the vector pRep5 (Groger et al. 1989) and mRNA from GM847, a human cell line with no known DNA repair defect. Sixty-one micrograms of mRNA were isolated from $2.6 \times 10^8$ cells by using an mRNA isolation kit (Fast Track; Invitrogen). Blunt-ended cDNAs then were synthesized from 10 μg of mRNA and an oligo(dT) primer containing a flanking *Not*I restriction site (Invitrogen), by using a modified Gubler-Hoffman protocol (Superscript; Gibco-BRL). The cDNA synthesis yielded 2.4 μg of double-stranded DNA. Two micrograms of cDNA then were ligated to *Sal*I adapters, cut with *Not*I, and selected by Sephacryl 500HR column chromatography (Pharmacia) for cDNAs of ≥700 bp. The 300 ng of cDNA that remained after size selection were ligated into *Not*I/*Xho*I-cut pRep5 and electroporated into DH10B *Escherichia coli.* The transformed bacteria were grown overnight, resulting colonies were harvested, and the library was amplified by overnight growth in Terrific Broth (Sambrook et al. 1989). The resulting library contained $>1.5 \times 10^6$ independent transformants, of which ~85% contain inserts with an average size of 1.6 kb.

### *Cell Culture, Transfection, and Selection*

GM5849 (AT5BIVI) was established from a complementation group D A-T patient, and GM847 was established from a patient with Lesch-Nyhan syndrome. Both are SV40-transformed fibroblast lines obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). AT4BINEI-155 is an SV40-transformed fibroblast line derived from a complementation group C A-T patient and was obtained from Dr. Colin Arlett (University of Sussex). GM5849-5L20 is a derivative of GM5849 containing a single integrated copy of the recombination vector pLrec (Herzing and Meyn, in press). All fibroblasts were grown in Dulbecco's minimal essential medium containing 15%–20% FBS. Fibroblast transfections were carried out as described elsewhere (Herzing and Meyn, in press), by using a Gene Pulser (Bio-Rad) set for 700 V and 25 μF. Each large-scale transfection of the cDNA library into GM5849-5L20 fibroblasts consisted of 13–21 individual transfections, each of which was plated onto a 35-cm culture dish. Stable transformants were selected by growth in 150 μg hygromycin (CalBiochem)/ml, beginning 48 h after electroporation, and were maintained by continuous growth in media containing hygromycin. Streptonigrin selection was carried out by adding streptonigrin to the media when the average size of transfected colonies reached 100 cells. Bacterial transformations were performed with a Gene Pulser according to a protocol supplied by Bio-Rad, by using settings of 17,500 V/cm, 25 μF, and 200 Ω.

### *Survival Assays*

Survival after exposure to streptonigrin was quantitated by plating cells in media containing various concentrations of drug. After 24 h, the medium was replaced, and cells were incubated for 10–14 d further. Colonies were fixed and stained with 0.4% Giemsa (Sigma), and sensitivity to streptonigrin was quantitated by counting colonies of ≥50 cells. Survival after X-ray exposure was determined by plating cells 3–4 h prior to irradiation and exposing cells to a 250-kV X-ray source (1.1 Gy/min). The medium was then replaced, and resultant colonies were counted after 10–14 d.

### Recombination Assays

For GM5849-5L20 and its transfected derivatives, spontaneous recombination rates between the mutant *lacZ* genes contained in the integrated pLrec recombination vector were measured by fluctuation tests, as described elsewhere (Herzing and Meyn, in press).

### Radio-resistant DNA Synthesis

Approximately $10^4$ cells were plated into media containing 0.01 μCi/[$^{14}$C]thymidine/ml (370 Bq/ml; specific activity $1.1 \times 10^9$ Bq/mmol; NEN) and were grown for 24 h; then the medium was removed, and the cells were washed and γ-irradiated with 0, 5, 10, or 20 Gy by using a $^{137}$Cs source (2.25 Gy/min). Fresh medium was added, and the cells were incubated another 2 h and then were pulse-labeled for 15 min with 10 μCi/ml [$^3$H]thymidine ($3.7 \times 10^5$ Bq/ml; specific activity $1.85 \times 10^{12}$ Bq/mmol; NEN). Cells were then harvested, and their DNA was extracted and counted as described elsewhere (Settleman et al. 1989).

### cDNA Rescue and Plasmid Analysis

Low-molecular-weight nuclear DNA containing episomal plasmids was extracted from fibroblasts by alkaline lysis (Belt et al. 1989) and electroporated into *E. coli* as described above. Restriction enzyme analysis, preparation of high-molecular-weight cellular DNA and $^{32}$P-labeled probes, gel electrophoresis, and Southern hybridization were as described elsewhere (Herzing and Meyn, in press). Sequencing of double-stranded cDNA inserts contained in the rescued pRep5 vectors was performed with primers to the polylinker region of pRep5 by using a commercial sequencing kit (USB). Northern hybridization was carried out as described by Sambrook et al. (1989), and quantitative analysis of northern blots was performed using a Molecular Dynamics PhosphoImager and ImageQuant software.

### cDNA Mapping

The pCAT4.2 cDNA was mapped by Southern hybridization of $^{32}$P-labeled pCAT4.2 cDNA to digests of genomic DNA from a human-rodent somatic cell hybrid mapping panel (Human Genetic Mutant Cell Repository, Camden, NJ). For fluorescent in situ hybridization (FISH), metaphase chromosomes were prepared after methotrexate synchronization and bromodeoxyuridine incorporation (Arnold et al., in press). cDNA probes were labeled with bio-11-dUTP (Sigma) by nick-translation (Brigati et al. 1983). Excess nucleotides were removed with a Sephadex G-50 spin column. In situ hybridization was performed essentially as described elsewhere (Lichter et al. 1990). Chromosomes were identified by DAPI staining (200 ng/ml). Digital images were obtained using a cooled CCD camera and a Zeiss epifluorescence microscope. FITC and DAPI signals were acquired as grey-scale images and were pseudocolored and merged prior to printing.

## Results

### cDNA Library Transfection of A-T Fibroblasts and Characterization of Primary Transformants

To isolate cDNAs that complement the phenotypic defects of the group D complementation group A-T fibroblast line GM5849-5L20, we undertook a series of four transfections with LN, a human cDNA library constructed using mRNA from SV40-transformed fibroblasts (GM847) and the EBV-based pRep5 expression vector (table 1). Sixty-eight of the ~610,000 primary transformants survived streptonigrin selection and were tested for resistance to 24 h exposure to 0.5 ng streptonigrin/ml. Forty-three were at least partially drug resistant, showing >20% survival after drug exposure, as compared with 1%–3% survival for the GM5849-5L20 parent, and some of these were nearly as drug resistant as the control cell line GM847 (data not shown). Figure 1*A* shows the results of a quantitative evaluation of streptonigrin resistance for three of these primary transformants and for appropriate controls. Streptonigrin-resistant primary transformants were screened for radiation resistance by determining their survival after exposure to 1.5, 3, and 5 Gy of 250-kV X-radiation. Fifteen streptonigrin-resistant transformants demonstrated >30% survival after 1.5 Gy irradiation and were classified as radiation resistant. Figure 1*B* shows survival after irradiation for control cell lines and for three streptonigrin-resistant transformants that arose independently on separate dishes. Several GM5849-5L20 transformants containing only pRep5, the parent vector, were also tested, and they were found to be as sensitive to streptonigrin and radiation as was GM5849-5L20 (data not shown).

GM5849-5L20, the A-T cell line used for our cDNA transfections, harbors a single copy of the recombination vector pLrec integrated into its genomic DNA. This vector contains two mutant copies of an SV40 promoter-driven *Eschrichia coli lacZ* gene that serve as recombination substrates. Recombination events involving the *lacZ* genes were detected by histochemical staining for β-galactosidase activity, and spontaneous intrachromosomal recombination rates were determined by fluctuation analysis. Table 2 summarizes re-

**Table 1**

**Transfections of an LN cDNA Library into GM4849-5L20 A-T D Fibroblasts**

| Transfer | Streptonigrin Treatment | LN cDNA Library DNA | | pRep5 Vector DNA | |
|---|---|---|---|---|---|
| | | No. of Stable Transformants[a] | No. of Surviving Colonies | No. of Stable Transformants[a] | No. of Surviving Colonies |
| I | .3 ng/ml, 15 d<br>.1 ng/ml, 7 d | 186,700 | 1 | 25,980 | 0 |
| II | .3 ng/ml, 13 d<br>.1 ng/ml, 7 d | 149,200 | 0 | 8,180 | 0 |
| III | .3 ng/ml, 6 d<br>.1 ng/ml, 11 d | 100,040 | 33 | 2,700 | 0 |
| IV | .3 ng/ml, 9 d<br>.1 ng/ml, 5 d<br>.3 ng/ml, 8 d<br>.1 ng/ml, 7 d | 174,300 | 34 | 4,000 | 0 |

[a] Total numbers of stable transformants were estimated by growing samples of cells from each electroporation, in hygromycin without streptonigrin.

sults for a control cell line (GM847), for the A-T cell line used for library transfections (GM5849-5L20), and for a derivative of GM5849-5L20 transfected with the pRep5 vector (5/p5/1a), as well as for streptonigrin-resistant primary transformants representing each of the rescued cDNAs species (see below). Transfection with the pRep5 vector alone only slightly decreased the spontaneous recombination rate of GM5849-5L20, while three of the primary transformants tested—CAT5-5, CAT4.5, and CAT4.4—had lost the hyperrecombination phenotype of their parent.

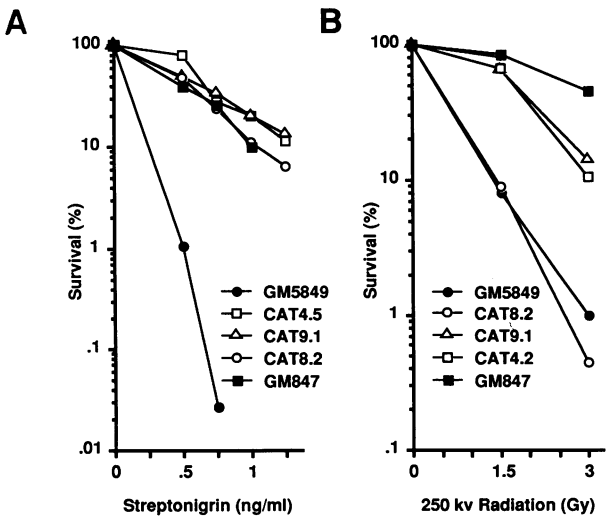


**Figure 1** Survival after (*A*) 24-h exposure to streptonigrin and (*B*) exposure to 250-kV ionizing radiation. CAT4.5, CAT8.2, CAT4.2, and CAT9.1 are A-T D fibroblasts transfected with the LN cDNA library; GM5849 is the parent A-T D cell line; and GM847 is a control cell line. Survival was measured by colony-forming ability. Points represent averages of duplicate plates.

### *cDNA Rescue and Analysis*

We attempted to rescue cDNAs by electroporating *E. coli* with low-molecular-weight nuclear DNA isolated from 29 of the streptonigrin-resistant primary transformants. Plasmids were recovered from all 29, but only 20 yielded vectors containing cDNA inserts. Analysis of the 20 cDNA-containing vectors by restriction enzyme digestion and Southern hybridization revealed that they represented 9 unrelated cDNAs ranging in size from 0.8 kb to 8.6 kb (data not shown). Four of the cDNAs were rescued from more than one independent transformant; for example, pCAT 4.2 was isolated from two drug-resistant clones of A-T fibroblasts that arose independently on separate dishes after transfection. Table 2 summarizes data concerning the rescued cDNAs and the phenotype of the primary transformants from which they were recovered. Primary transformants harboring the same cDNA were concordant for phenotype (data not shown) and were classifiable into two broad groups: (1) streptonigrin-resistant radiosensitive cells with high spontaneous rates of recombination (e.g., CAT8.2) and (2) streptonigrin-resistant radio-resistant cells with near normal rates of recombination (e.g., CAT4.5).

**Table 2**

**Characteristics of Primary Transformants and the cDNAs That They Contain**

| Group | Size of Rescued cDNA (kb) | No. of Independent Rescues[a] | Proportion of Rescued cDNAs Containing Inserts | Cellular Phenotype of Primary Transformants: Streptonigrin Resistance | Cellular Phenotype of Primary Transformants: Radiation Resistance | Cellular Phenotype of Primary Transformants: Recombination Rate[b] ($\times 10^{-6}$ events/cell generation) | Chromosomal Location |
|---|---|---|---|---|---|---|---|
| Control cell lines: | | | | | | | |
| GM847 | | | | | ++++ | ++++ | 7 |
| GM5849-5L20 | | | | | − | − | 1650 |
| 5L20-5/p5/1a | | | | | − | − | 770 |
| Primary transformants: | | | | | | | |
| CAT5.2 | 2.7 | 1 | 19/19 | ++++ | − | 270 | |
| CAT8.2 | 2.1 | 2 | 15/93 | ++++ | − | 560 | |
| CAT5-3 | 1.7 | 1 | 12/12 | ++++ | − | ND | 7p13 |
| CAT4-2 | .8 | 1 | 19/19 | ++++ | − | ND | |
| CAT4.2 | 1.9 | 2 | 6/32 | ++++ | ++ | ND | 6 |
| CAT5-5 | 1.4 | 1 | 1/1 | ++++ | ++ | 15 | 15q22 |
| CAT4.5 | 4.5 | 2 | 18/35 | ++++ | ++ | 9.1 | 17p11.1 |
| CAT4.4 | 4.5 | 9 | 42/289 | ++++ | ++ | 6.2 | |
| CAT6-4 | 8.6 | 1 | 1/7 | ++++ | ++ | ND | 18q21 |

[a] No. of rescued plasmids carrying an insert/total no. of plasmids analyzed for each primary transformant or groups of primary transformants yielding the same cDNA.

[b] ND = not determined.

### *Partial Sequencing and Mapping of cDNAs*

In order to identify the rescued cDNAs, we sequenced the initial 200–250 bp of each insert and compared its sequence with sequences in the GenBank (release 72) and EMBL (release 31) databases, using the FASTA homology search. Two cDNAs shared homology with known genes. There were no discernible mismatches between the 243 bp of pCAT4.2 sequenced and the *dek* cDNA (von Lindern et al. 1992), with the pCAT 4.2 cDNA insert beginning 930 bases into the *dek* coding region, while the 234 bp of pCAT5-3 sequenced is >99% identical with the human insulin growth factor binding protein 3 (IGFBP-3) beginning 676 bases into the coding region of the gene (Wood et al. 1988) (data not shown).

The pCAT4.2 insert was hybridized to a human-rodent somatic cell hybrid mapping panel and unambiguously mapped to chromosome 6 (data not shown). Four of the remaining cDNAs were successfully mapped by FISH: pCAT4.5 mapped to 17p11.1, pCAT6-4 mapped to 18q21, pCAT5-3 mapped to 7p13, and pCAT5-5 mapped to 15q22 (data not shown). Although pCAT4.5 mapped to 17p, a p53 cDNA probe did not hybridize to pCAT4.5 (data not shown). pCAT4.4 could not be mapped, because of repetitive sequences present in the cDNA insert.

Northern analysis of RNA obtained from the control fibroblast line GM847 and from the A-T D fibroblast line GM5849 was performed using the pCAT4.5 cDNA as a $^{32}$P-labeled probe. A single, ~4.5-kb transcript was detected in samples of RNA isolated from the two cell lines after an overnight exposure of X-ray film to the northern blot (fig. 2). A subsequent 1-wk exposure did not reveal additional pCAT4.5-related transcripts (data not shown). Northern blots were quantitatively analyzed using a PhosphoImager, and, in triplicate samples, RNA isolated from the A-T D line was found to contain an average of 2.0-fold (range 1.7- to 2.7-fold) more pCAT4.5 mRNA than did RNA isolated from the control cell line.

### *Phenotypic Characterization of Secondary Transformants*

To verify the ability of rescued cDNAs to complement A-T D cells, individual cDNAs were transfected into GM5849-5L20 fibroblasts, and stable transfor-

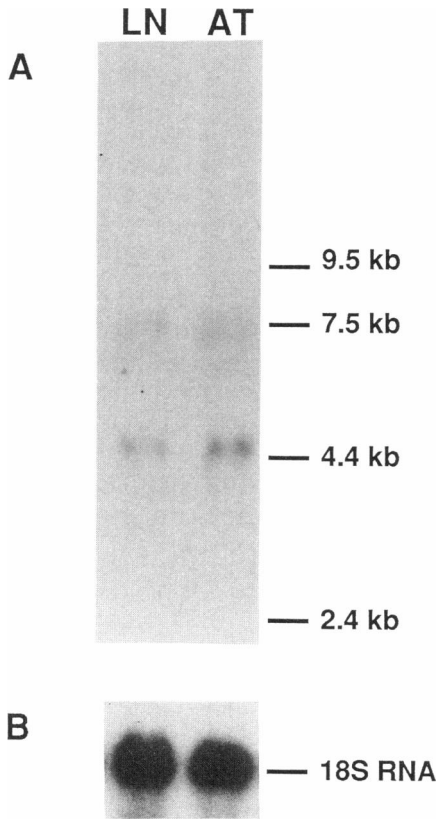


**Figure 2** Northern analysis of pCAT4.5 expression in the control fibroblast line GM847 and the A-T D fibroblast line GM5849. Ten micrograms of total cellular RNA were loaded into each lane. LN = GM847; and AT = GM5849. *A*, X-ray film exposed overnight to the membrane after hybridization of blotted RNA to a $^{32}$P-labeled probe made using the pCAT4.5 cDNA insert. *B*, X-ray film exposed overnight to the same membrane after hybridization of blotted RNA to a $^{32}$P-labeled probe specific for 18S RNA. The intensity of the 18S RNA bands in the two lanes varied by <5%, as determined by PhosphoImager analysis, indicating that the amount of RNA transferred to the membrane was the same in both lanes.

mants were tested for streptonigrin and/or radiation resistance. In all, 5/8 pCAT4.4 transformants, 3/7 pCAT5-5 transformants, 2/3 pCAT5.2 transformants, 3/4 pCAT4.2 transformants, and 6/10 pCAT4.5 transformants were partially resistant to streptonigrin and/or radiation exposure (data not shown). All transformants were concordant for either resistance or sensitivity to both agents. Survival after exposure to streptonigrin is shown, in figure 3*A*, for representative streptonigrin-resistant secondary transformants of pCAT4.2 and pCAT4.5, while figure 3*B* documents the partial radiation resistance of these transformants. pCAT4.5 was also transfected into AT4BINEI-155, a fibroblast line derived from a complementation group C A-T patient. Six stable transformants were examined for streptonigrin sensitivity. In contrast to the acquisition of streptonigrin resistance by the majority of pCAT4.5 transformants of A-T D fibroblasts, none of the six independently derived A-T C pCAT4.5 transformants were streptonigrin resistant (data not shown). The differential ability of pCAT4.5 to complement 6 of 10 secondary transformants of a group D fibroblast line but 0 of 6 secondary transformants of a group C fibroblast line was statistically significant in a 2 × 2 contingency-table analysis ($\chi^2 = 5.76$; df = 1; $P < .017$).

pCAT4.2 and pCAT4.5 were tested for their ability to complement two other phenotypic defects of A-T fibroblasts. The high spontaneous recombination rate of the A-T D cell line was complemented by transfection with pCAT4.2 or pCAT4.5 (table 3). We also determined the effect of these two cDNAs on radio-resistant DNA synthesis, a consequence of the $G_1$/S cell-cycle checkpoint defect in A-T (Kastan et al. 1992). As can be seen in figure 4, pCAT4.5/9a and pCAT4.2/4d—which are complemented secondary transformants of pCAT4.2 and pCAT4.5, respectively—have lost the radio-resistant DNA synthesis of their parent A-T D line, GM5849-5L20.

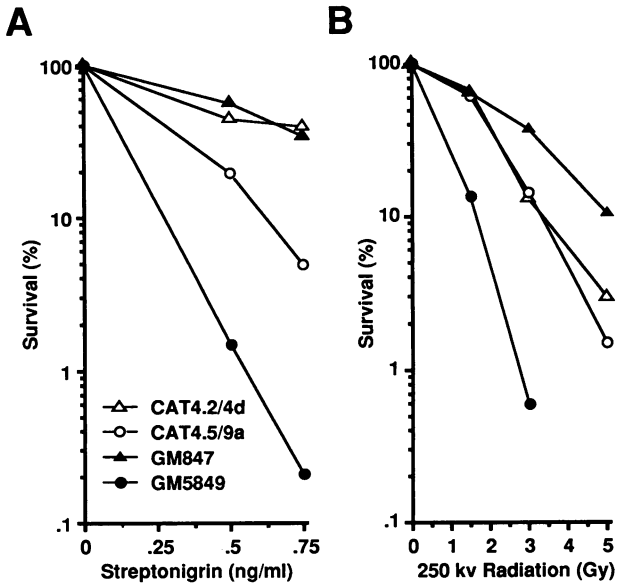


**Figure 3** Survival after (*A*) 24-h exposure to streptonigrin and (*B*) exposure to 250-kV ionizing radiation. CAT4.2/4d and CAT4.5/9a are A-T D fibroblasts transfected with the pCAT4.2 and pCAT4.5 cDNAs, respectively; GM5849 is the parent A-T D cell line; and GM847 is a control cell line. Survival was measured by colony-forming ability. Points represent averages of duplicate plates.

**Table 3**

**Spontaneous Intrachromosomal Recombination Rates of *lacZ* Genes in Human Fibroblasts Containing pLrec**

| Cell Line | Total No. of LacZ$^+$ Cells Detected | Total No. of Cells Screened | Rate of Conversion to LacZ$^+$ ($\times 10^{-5}$ conversions/ cell generation) |
|---|---|---|---|
| Control cell line: | | | |
| GM847-LNL6 | 206 | $7.6 \times 10^6$ | .7 |
| A-T cell line: | | | |
| GM5849-5L20 | 50 | $9.23 \times 10^3$ | 189 |
| GM5849-5L20 transformants: | | | |
| CAT4.2/4d | 40 | $2.84 \times 10^5$ | 5.2 |
| CAT4.5/9a | 73 | $4.37 \times 10^5$ | 5.1 |

## Discussion

By transfecting the immortalized A-T D fibroblast line GM5849-5L20 with LN, a human cDNA library constructed using the EBV-based episomal expression vector pRep5, we achieved stable transfection frequencies of 0.5%–2%. This is 50–100-fold better than transfection frequencies obtained when GM5849 is transformed with genomic DNA (Lohrer et al. 1988) and demonstrates the high transfection efficiency possible when episomal vectors are used. Selection against streptonigrin sensitivity resulted in survival of only 68 of ~610,000 stable LN transformants but allowed recovery of clones that had been complemented for several aspects of the A-T phenotype. Forty-three of these 68 colonies demonstrated streptonigrin resistance on further testing. Fifteen of the streptonigrin-resistant transformants also exhibited partial correction of radiation sensitivity, and those radiation-resistant clones that were tested had correction of high spontaneous recombination rates. Correction of the A-T phenotype was not due to the presence of pRep5 vector sequences.

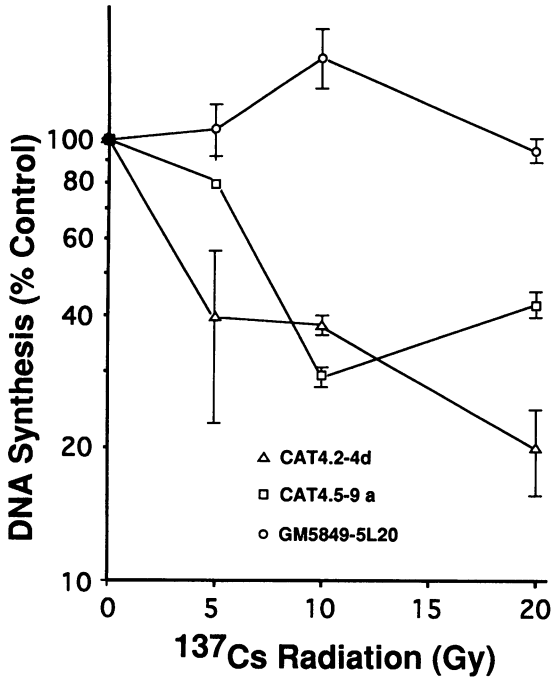


**Figure 4** Inhibition of DNA synthesis rate, as a function of radiation dose. CAT4.2/4d and CAT4.5/9a are GM5849-5L20 fibroblasts transfected with the pCAT4.2 and pCAT4.5 cDNAs, respectively; and GM5849-5L20 is the parent A-T D cell line. Error bars indicate ranges of duplicate cultures.

An advantage of EBV-based episomal vectors over transfection of genomic DNA for complementation cloning is the relative ease in rescuing complementing sequences. From 20 of 29 streptonigrin-resistant clones, we successfully rescued plasmids containing cDNAs. These 20 represented nine unrelated cDNAs, including several that were rescued multiple times from independently arising clones. Only five of the nine cDNAs came from streptonigrin-resistant clones that also had acquired partial radiation resistance, including two that expressed normal rates of intrachromosomal recombination. Clones that were streptonigrin resistant but radiation sensitive may have acquired cDNAs that interfered with the uptake, metabolism, or action of streptonigrin, rather than cDNAs that complemented the underlying defect in A-T cells.

Previous reports by Green et al. (1987) and Kapp and Painter (1989) described isolation of primary transformants that, after transfection of genomic human DNA into A-T D fibroblasts, had acquired partial resistance

to killing by irradiation but still expressed the radio-resistant DNA synthesis of the parent A-T line. Kapp et al. (1992) recently isolated an ~40-kb fragment of human genomic DNA from one of these radiation-resistant primary transformants that, on transfection into A-T D fibroblasts, was found to partially correct their radiosensitivity. We also obtained radio-resistant primary transformants after transfection of A-T D fibroblasts, and we have gone on to isolate five human cDNAs (pCAT4.4, pCAT5-5, pCAT5.2, pCAT4.2, and pCAT4.5) that confer mutagen resistance to A-T D cells on secondary transformation. These are the first human cDNAs reported to complement the mutagen sensitivity of A-T cells.

The five complementing cDNAs conferred mutagen resistance to a majority—but not all—of the secondary A-T D transformants. The proportion of secondary transformants that acquired mutagen resistance is similar to that reported for other cDNAs that complement DNA repair defects in mammalian cells (Weber et al. 1988; Tanaka et al. 1990) and compares favorably with the results of Kapp et al. (1992), who found that a complementing genomic fragment conferred partial radio resistance in only 3 of 50 stable transformants of the AT5BIVI A-T D fibroblast line. The inability of complementing cDNAs to confer mutagen resistance to all secondary transformants may be the result of either rearrangement of the vector or damage sustained during transfection (Wake et al. 1984).

We tentatively identified one of the cDNAs, pCAT4.2, as a fragment of the *dek* gene, on the basis of map location (chromosome 6) and complete identity between the 243 bp of pCAT4.2 sequenced to date and the *dek* cDNA sequence. The *dek* gene was isolated recently because of its involvement, along with the *can* gene, in a consistent t(6;9)(p23;q34) translocation breakpoint in certain acute myeloid leukemias. This translocation results in the expression of a leukemia-specific protein from a chimeric gene that consists of the 5′ control elements and most of the *dek* gene's coding region fused to the 3′ half of coding region of the *can* gene (von Lindern et al. 1992). Two-thirds of the *dek* sequence contained in pCAT4.2 also is included in the *dek-can* chimeric gene. Although the *dek-can* chimeric gene may function as an oncogene, little is known about the normal *dek* gene, other than that it is widely expressed and that the DEK protein appears to be associated with chromatin (von Lindern et al. 1992).

The pCAT4.5 clone contains a 4.5-kb cDNA that maps to 17p11.1, near but centromeric to the p53 gene. On the basis of the results of restriction enzyme analysis, Southern hybridization, and partial sequencing, it neither is the p53 gene nor has any homology to sequences in the GenBank and EMBL databases. The complementing cDNAs were derived from mRNAs isolated from cells that had not been treated with ionizing radiation or chemical mutagens, indicating that, at least in transformed fibroblast lines, there is a constitutive level of expression of these cDNAs. Initial northern analysis of pCAT4.5 mRNA expression in a control and an A-T fibroblast line confirmed constitutive expression of the pCAT4.5 gene. The similar sizes of the single pCAT4.5 mRNA transcript and of the pCAT4.5 cDNA suggest that the pCAT4.5 cDNA may represent a full-length transcript of the pCAT4.5 gene.

Our isolation of five unrelated complementing cDNAs suggests that there are multiple ways in which to suppress the A-T D phenotype and demonstrates that phenotypic complementation is insufficient for establishing complementing cDNAs as disease genes for A-T. The underlying defect in A-T is unknown, although abnormalities of DNA repair, genetic recombination, chromatin structure, and cell-cycle checkpoints have been proposed (reviewed in Meyn, submitted). Kastan et al. (1992) recently suggested that the A-T gene product(s) functions upstream of p53 in a multistep DNA damage-responsive pathway that leads to cell-cycle arrest at the $G_1$/S border and that A-T homozygotes are unable to activate the $G_1$/S cell-cycle checkpoint after exposure to ionizing radiation, thereby resulting in radio-resistant DNA synthesis. A-T cells also lack the $G_2$/M DNA damage-sensitive cell-cycle checkpoint, do not exhibit enhanced repair and mutagenesis of irradiated viruses, and may have an unusually low threshold for activating programmed cell death in the face of DNA damage (Zampetti-Bosseler and Scott 1981; Hilgers et al. 1987, 1989; Bates and Lavin 1989; Schimke et al. 1991; Meyn, submitted). The apparent lack of multiple cellular responses to DNA damage in A-T homozygotes has led one of us to propose that the A-T gene product(s) normally plays a critical role in a signal transduction network that, in response to spontaneous and induced DNA damage, activates a group of cellular functions designed to facilitate repair of DNA damage, prevent genetic instability, and promote survival (Meyn, submitted). One branch of this network is the p53-dependent damage-sensitive $G_1$/S checkpoint pathway outlined by Kastan et al. (1992).

The pCAT4.2 fragment of the *dek* gene and the pCAT4.5 cDNA partially complement three different aspects of the A-T phenotype—i.e., mutagen sensitiv-

ity, high spontaneous recombination rate, and radioresistant DNA synthesis—thereby lending support to these and other A-T models in which the A-T gene product(s), rather than playing a direct enzymatic role in DNA repair or recombination, mediates multiple responses to DNA damage. The genes represented by pCAT4.2 and pCAT4.5 may be involved in an A-T damage-surveillance network, perhaps at a step downstream of the site of A-T gene action. Alternatively, overexpression of the pCAT4.2 or pCAT4.5 cDNAs may facilitate functioning of the defective A-T D gene product. Study of *dek* and pCAT4.5 expression and function in normal and A-T cells, as well as determination of the effect of expression of full-length *dek* cDNAs on the A-T phenotype, may help to delineate the function of these genes. Neither pCAT4.2 nor pCAT4.5 completely complemented the A-T defects, although the extent of complementation of radiation sensitivity is similar to that reported for the chromosome 11q23 cosmid isolated by Kapp et al. (1992). This may be the result of having obtained only partial clones, rearrangement of the cDNA inserts, or unregulated expression from the RSV promoter of the pRep5 vector. Although a useful characteristic for many purposes, the constitutive strong promoters used in pRep5 and other EBV-based cDNA expression vectors could interfere with cloning of A-T genes if these genes are involved in a regulatory network that controls cell-cycle progression.

It is possible that, instead of being a secondary suppressor, one of the complementing cDNAs may represent the A-T D disease gene itself. pCAT4.5 is a particularly attractive candidate, in that it complements three different phenotypic abnormalities in A-T D fibroblasts but does not complement the mutagen sensitivity of an A-T C fibroblast line. Hence complementation by pCAT4.5 appears to be specific for A-T D, as might be expected for the A-T D disease gene. Although northern analysis of RNA obtained from the GM5849 A-T D fibroblast line did not indicate a gross difference in the size of pCAT4.5 mRNA when compared with that of a control fibroblast line, constitutive levels of pCAT4.5 mRNA averaged twofold higher in the A-T D fibroblasts. Altered expression of the pCAT4.5 gene in A-T D cells, as suggested by this initial northern analysis, is consistent with the possibility that pCAT4.5 may be the A-T D gene.

The pCAT4.5 cDNA maps to 17p11. It has been proposed (Kapp et al. 1992; Sobel et al. 1992) that the A-T D gene is located on 11q23, telomeric to the A-T A and C loci. If this location were correct, then pCAT4.5, as well as our other mapped cDNAs, could be eliminated from further consideration as the A-T D gene, on the basis of map position alone. The map assignment of A-T D to 11q23 is based, in part, on studies finding that fragments of 11q can complement the radiosensitivity and cell-cycle abnormalities of group D fibroblasts (Ejima et al. 1990; Lambert et al. 1991) and on the demonstration that a cosmid containing human genomic DNA that maps to 11q23 can partially complement the radiation sensitivity of A-T D fibroblasts (Kapp et al. 1992). However, our isolation of multiple unrelated complementing cDNAs from different regions of the genome demonstrates that phenotypic complementation cannot be used as evidence for determining the chromosomal location of the group D A-T gene. The remaining evidence cited for the proposed assignment of the A-T D locus to 11q23 is indirect; several linkage studies that included non-A and non-C complementation group families in their analyses yielded no evidence of A-T loci located outside the 11q23 region (e.g., Sanal et al. 1990), and an analysis of international consortium data (Sobel et al. 1992) found that location-score maps for the 11q23 region demonstrate a weakly bimodal distribution, consistent with the presence of a second A-T locus telomeric to the previously identified group A/group C locus. However, there have been no reported linkage studies that included known group D families. In addition, a recent report by Hernandez et al. (1993) provides evidence for the existence of A-T loci outside 11q23, by excluding linkage to 11q23 markers in an A-T family whose complementation group is unknown. We conclude that the map position of A-T D is far from certain at the present time, and we consider pCAT4.5 to represent a candidate gene for A-T group D. Further study of pCAT4.5, including sequencing of the gene and additional analysis of its expression in both normal and A-T patients, may demonstrate functionally significant sequence and/or expression abnormalities in A-T D homozygotes that then would establish its identity as the A-T D disease gene.

Combining episomal expression-vector complementation with FISH mapping of rescued cDNAs offers a rapid and efficient method for obtaining candidate A-T disease genes, as well as secondary suppressors that could shed light on both the nature of the A-T defect and the cellular processes that it affects. Our approach can be adapted readily for use with other A-T complementation groups. Multiple attempts to clone human DNA repair genes by complementing the mutagen sensitivity of A-T and other human diseases by using genomic DNA transfection have succeeded only rarely

(Green et al. 1987; Lohrer et al. 1988; Kapp and Painter 1989). EBV-based episomal expression vectors were designed to circumvent problems associated with genomic DNA transfection. The present study, along with recent reports on the cloning of candidate XP and FA genes by using EBV-based episomal expression vectors (Legerski and Peterson 1992; Strathdee et al. 1992), documents the utility of this approach in isolating putative DNA repair genes and suggests that these vectors may facilitate the cloning of cDNAs that complement defects in mutant human cells for which there is strong selection or sorting.

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## References

Arnold N, Bhatt M, Ried T, Ward DC, Wienberg J. In: Kessler C (ed) Techniques and methods in molecular biology: non-radioactive labeling and detection of biomolecules. Springer, Berlin (in press)

Bates PR, Lavin MF (1989) Comparison of gamma-radiation-induced accumulation of ataxia telangiectasia and control cells in G2 phase. Mutat Res 218:165–170

Belt PB, Groenevelt H, Teubel WJ, van de Putte P, Backendorf C (1989) Construction and properties of an Epstein-Barr-virus-derived cDNA expression vector for human cells. Gene 84:407–417

Brigati DJ, Meyerson D, Leary JJ, Spalholtz B, Travis SZ, Fong CKY, Hsiung GD, et al (1983) Detection of viral genomes in cultured cells and paraffin-embedded tissue sections using biotin-labeled hybridization probes. Virology 126:32–50

Cohen MM, Levy HP (1989) Chromosome instability syndromes. Adv Hum Genet 18:43–149

Ejima Y, Oshimura M, Sasaki MS (1990) Establishment of a novel immortalized cell line from ataxia-telangiectasia fibroblasts and its use for the chromosomal assignment of radiosensitivity gene. Int J Radiat Biol 58:989–997

Green MH, Lowe JE, Arlett CF, Harcourt SA, Burke JF, James MR, Lehmann AR, et al (1987) A gamma-ray-resistant derivative of an ataxia telangiectasia cell line obtained following DNA-mediated gene transfer. J Cell Sci Suppl 6:127–137

Groger RK, Morrow DM, Tykocinski ML (1989) Directional antisense and sense cDNA cloning using Epstein-Barr virus episomal expression vectors. Gene 81:285–294

Hecht F, Hecht BK (1990) Cancer in ataxia-telangiectasia patients. Cancer Genet Cytogenet 46:9–19

Hernandez D, McConville CM, Stacey M, Woods CG, Brown MM, Shutt P, Rysiecki G, et al (1993) A family showing no evidence of linkage between the ataxia telangiectasia gene and chromosome 11q22-23. J Med Genet 30:135–140

Herzing LBK, Meyn MS. Novel *lacZ*-based vectors for studying genetic recombination in mammalian cells. Gene (in press)

Hilgers G, Abrahams PJ, Chen YQ, Schouten R, Cornelis JJ, Lowe JE, van der Eb AJ, et al (1989) Impaired recovery and mutagenic SOS-like responses in ataxia telangiectasia cells. Mutagenesis 4:271–276

Hilgers G, Chen YQ, Cornelis JJ, Rommelaere J (1987) Deficient expression of enhanced reactivation of parvovirus H-1 in ataxia telangiectasia cells irradiated with X-rays or u.v. light. Carcinogenesis 8:315–319

Hoeijmakers JHJ, Odijk H, Westerveld A (1987) Differences between rodent and human cell lines in the amount of integrated DNA after transfection. Exp Cell Res 169:111–119

Kapp LN, Painter RB (1989) Stable radioresistance in ataxia-telangiectasia cells containing DNA from normal human cell. Int J Radiat Biol 56:667–675

Kapp LN, Painter RB, Yu L-C, van Loon N, Richard CW III, James MR, Cox DR, et al (1992) Cloning of a candidate gene for ataxia-telangiectasia group D. Am J Hum Genet 51:45–54

Kastan MB, Zhan Q, el Deiry WS, Carrier F, Jacks T, Walsch WV, Plunkett BS, et al (1992) A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. Cell 71:587–597

Lambert C, Schultz RA, Smith M, Wagner-McPherson C, McDaniel LD, Donlon T, Stanbridge EJ, et al (1991) 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. Proc Natl Acad Sci USA 88:5907–5911

Legerski R, Peterson C (1992) Expression cloning of a human DNA repair gene involved in xeroderma pigmentosum group C. Nature 359:70–73

Lichter P, Tang CJ, Call K, Hermanson G, Evans GA, Housman D, Ward DC (1990) High-resolution mapping of human chromosome 11 by in situ hybridization with cosmid clones. Science 247:64–68

Lohrer H, Blum M, Herrlich P (1988) Ataxia telangiectasia resists gene cloning: an account of parameters determining gene transfer into human recipient cells. Mol Gen Genet 212:474–480

Mayne LV, Jones T, Dean SW, Harcourt SA, Lowe JE, Priest-

ley A, Steingrimsdottir H, et al (1988) SV 40-transformed normal and DNA-repair-deficient human fibroblasts can be transfected with high frequency but retain only limited amounts of integrated DNA. Gene 66:65–76

Meyn MS (1993) High spontaneous rates of intrachromosomal recombination in ataxia-telangiectasia. Science 260: 1327–1330

———. Ataxia telangiectasia, cell cycle checkpoints and programmed cell death in the cellular response to DNA damage (submitted)

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Sanal O, Wei S, Foroud T, Malhotra U, Concannon P, Charmley P, Salser W, et al (1990) Further mapping of an ataxia-telangiectasia locus to the chromosome 11q23 region. Am J Hum Genet 47:860–866

Schimke RT, Kung AL, Rush DF, Sherwood SW (1991) Differences in mitotic control among mammalian cells. Cold Spring Harb Symp Quant Biol 56:417–425

Sedgwick RP, Boder E (1991) Ataxia-telangiectasia. Handbook Clin Neurol 16:347–423

Settleman J, Fazeli A, Malicki J, Horwitz BH, DiMaio D (1989) Genetic evidence that acute morphologic transformation, induction of cellular DNA synthesis, and focus formation are mediated by a single activity of the bovine papillomavirus E5 protein. Mol Cell Biol 9:5563–5572

Sobel E, Lange E, Jaspers NGJ, Chessa L, Sanal O, Shiloh Y, Taylor AMR, et al (1992) Ataxia-telangiectasia: linkage evidence for genetic heterogeneity. Am J Hum Genet 50:1343–1348

Strathdee CA, Gavish H, Shannon WR, Buchwald M (1992) Cloning of cDNAs for Fanconi's anaemia by functional complementation. Nature 356:763–767

Swift M, Reitnauer PJ, Morrell D, Chase CL (1987) Breast and other cancers in families with ataxia-telangiectasia. N Engl J Med 316:1289–1294

Tanaka K, Miura N, Satokata I, Miyamoto I, Yoshida MC, Satoh Y, Kondo S, et al (1990) Analysis of a human DNA excision repair gene involved in group A xeroderma pigmentosum and containing a zinc-finger domain. Nature 348:77–79

von Lindern M, Fornerod M, van Baal S, Jaegle M, de Wit T, Bulis A, Grosveld G (1992) The translocation (6;9), associated with a specific subtype of acute myeloid leukemia, results in the fusion of two genes, dek and can, and the expression of a chimeric, leukemia-specific dek-can mRNA. Mol Cell Biol 12:1687–1697

Wake CT, Gudewicz T, Porter T, White A, Wilson JH (1984) How damaged is the biologically active subpopulation of transfected DNA? Mol Cell Biol 4:387–398

Weber CA, Salazar EP, Steward SA, Thompson LH (1988) Molecular cloning and biological characterization of a human gene, ERCC2, that corrects the nucleotide excision repair defect in CHO UV5 cells. Mol Cell Biol 8:1137–1146

Wood WI, Cachianes G, Henzel WJ, Winslow GA, Spencer SA, Hellmiss R, Martin JL, et al (1988) Cloning and expression of the growth hormone-dependent insulin-like growth factor-binding protein. Mol Endocrinol 2:1176–1185

Zampetti-Bosseler F, Scott D (1981) Cell death, chromosome damage and mitotic delay in normal human, ataxia telangiectasia and retinoblastoma fibroblasts after x-irradiation. Int J Radiat Biol 39:547–558