Altered splicing of the ATDC message in ataxia telangiectasia group D cells results in the absence of a functional protein

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The ATDC gene was cloned using functional complementation and complements the radiosensitivity of ataxia telangiectasia (AT) group D cells. Although a number of transcripts have been detected, only a 3.0 kb cDNA found in a HeLa cell cDNA library has been cloned. Since AT group D cells express only a 2.4 kb transcript, efforts were made to clone and sequence this transcript. Using a biotinylated oligonucleotide probe, mRNA preparations were enriched in ATDC-related sequences. After this enrichment, 2.4 kb clones were obtained from the resulting library. The 2.4 kb transcript appears to be untranslated, since no protein from this transcript has been detected in AT group D cells, and this transcript is probably non-functional, since a splicing variation has positioned part of intron 1 near the first methionine codon in exon 1, eliminating most of exon 1 and important functional regions from this transcript. This transcript now has a stop codon located 33 bp in front of the first methionine, which would stop translation after the eleventh amino acid. As a result of these changes, the AT group D cell line (AT5BI) expresses no functional ATDC protein.

Introduction

The ATDC gene complments radiation sensitivity in AT5BI cells, an ataxia telangiectasia (AT) complementation group D cell line. It has a complex expression pattern and up to nine different transcripts have been detected in various cell lines (Hosoi and Kapp, 1994). ATDC cDNA was cloned from a HeLa cDNA library (Kapp et al., 1992), but only a 3.0 kb transcript was obtained from these efforts. Previous work (Hosoi and Kapp, 1994) showed that AT group D cells (AT5BI) expressed only a 2.4 kb transcript of ATDC. However, previous efforts to clone this particular ATDC transcript failed. This transcript is of great interest since it is apparently the only form of the ATDC gene expressed in the AT5BI cell line, which is a group D cell line and which was used for the functional complementation experiments which resulted in cloning of the ATDC gene (Kapp and Painter, 1989; Kapp et al., 1992). The 3.0 kb transcript of the ATDC gene complemented the radiosensitivity of these group D cells, suggesting that it was a candidate for the AT group D gene. However, no mutations were found in the genomic sequences of the 3.0 kb transcript in AT group D cells (Leonhardt et al., 1994).

Since AT5BI group D cells express only the ATDC 2.4 kb transcript, andthis was not cloned in previous work, the aim of the work described here was to clone this 2.4 kb ATDC transcript expressed in AT group D cells and to sequence it to determine if it could produce a functional protein.

Materials and methods

Cell lines

HS27 cells are a normal human diploid fibroblast cell strain obtained from the Cell Culture Facility at UCSF. AT5BIA group D transformed cells (GMS849) were used as a source for mRNA. These cells are also called TAT5 cells. These cells were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). Cells were grown in MEM supplemented with 15% fetal calf serum, glutamine (Gibco) and penicillin/streptomycin (Gibco).

ATDC9 cells are AT5BIA cells complemented with the ATDC gene in an expression vector and which have a near normal radiosensitivity.

Construction of an enriched ATDC cDNA library

An AT5BI cDNA library was constructed using conventional methods (Sambrook et al., 1989). More than 5×106 primary plaques were screened using a full-length 3.0 kb ATDC cDNA as probe. However, the 2.4 kb ATDC transcript was not present in the library. In order to find this apparently rare transcript, another method of constructing a cDNA library was tried. To construct a cDNA library which contains a high percentage of ATDC cDNA, the protocol used with the Cap-finder cDNA library construction kit from Clontech was modified. The kit protocol is based on the use of a 5'-cap oligonucleotide and long distance PCR. A hybridization step was added after the first strand synthesis in order to concentrate the rare ATDC-related transcripts. Biotinylation of the oligonucleotide was achieved by biotin-14-dCTP addition to the 3'-end of the products with terminal deoxynucleotidyl transferase.

Poly(A) RNA was isolated from AT5BI cells using a Fast Track mRNA isolation kit (Invitrogen) and the first DNA strand was synthesized using MMLV reverse transcriptase with an oligo(dT) primer and a 5'-oligonucleotide and long distance PCR. After LD-PCR with a 5'-oligonucleotide from an ATDC exon 2 sequence (5'-GCCACTT-CCTCAGCTTCATCCTCAA-3'). This sequence was selected for concentration of ATDC sequences for several reasons. Previous data showed that very low levels of the ATDC mRNA containing exons 3–8 should be present, even in SV40-transformed AT group D cells (Hosoi and Kapp, 1994), and exon 2 and the 5'-region of exon 3 encode a leucine zipper that may be an important functional region for ATDC protein (Leonhardt et al., 1994).

Hybridization was performed in 6X SSC, 0.1% SDS and 5X Denhardt's solution at 48°C for 1 h. At the end of the hybridization, streptavidin-coated magnetic beads (Dynabeads M-280 streptavidin; Dynal) were added to the solution. The mixture was incubated at room temperature for 30 min and the beads were collected with a magnet. The collected beads were washed three times with a washing solution consisting of 0.1X SSC, 0.1% SDS and 1 mM EDTA. Hybridized single-strand DNAs were eluted from the beads by incubation in an elution solution (50 mM NaOH, 1 mM EDTA). Eluted DNA was neutralized by the addition of one half volume of 7.5 M ammonium acetate and then ethanol precipitated. The pellet was dissolved in 15 μl of deionized, Milli-Q (Millipore)-treated sterile water and used as a template for a long distance PCR reaction (LD-PCR). After LD-PCR with a 5'-cap oligonucleotide and an oligo(dT) primer for 25 cycles, double-stranded cDNA was blunted with a T4 DNA polymerase reaction and ligated with EcoRI/NotI/SalI adapters (Clontech). The cDNA was then phosphorylated, ligated with KpnI vector arms, packaged with a Gigapack Gold kit (Stratagene) and transformed into host Escherichia coli strain Y1009R-. The average titer of the primary cDNA libraries was 0.5–1.0×109 p.f.u. and the percentage of recombinants was ~90%.

Library screening

Full-length (3018 bp) ATDC cDNA was used as a probe for detecting the ATDC message from AT5BI cells. Hybridization was performed in a solution
This approach efficiently enriched the library in ATDC-positive clones, which were normally present at very low concentrations. From the enriched library, nine ATDC-positive clones were obtained out of a total of $2.5 \times 10^4$ plaques, or one positive clone per $2.7 \times 10^3$ plaques. Out of these nine clones, five contained a 2.4 kb insert, one clone contained a 2.0 kb insert and the remaining four clones contained inserts which were approximately 1.0 kb in length but did not contain the 3'-region of ATDC.

The sequence of the new 2.4 kb clone was compared with the intron 1 region near exon 2 in the K1 cosmid, which contains the 5'-half of the ATDC genomic sequence (Kapp et al., 1992). This comparison showed that a part of intron 1 just upstream of exon 2 had been spliced into the 3'-region of exon 1 just 5' to the methionine codon (Figure 1). This splicing variation results in the removal of most of exon 1 and its replacement with a part of intron 1 from the genomic sequence upstream of exon 2 in AT5BIVA AT group D cells.

Northern blot analysis using the ATDC exon 1 sequence as a probe detected no 2.4 kb bands in diploid AT5B1 group D cells (data not shown). This indicated that the 2.4 kb message in AT5B1 group D cells does not contain exon 1 of the 3018 bp ATDC transcript. Additional northern blot analysis was performed using a full-length ATDC cDNA as a probe (Figure 2). With the full-length cDNA probe, 2.4 kb bands can be seen in normal HS27 cells and in ATDC9 cells. AT5B1 cells show a slightly smaller ATDC band, but the ATDC transcript in these cells does not contain exon 1. Transformed AT5B1 cells (TAT5 or AT5BIVA cells) have no detectable ATDC transcript. ATDC9 cells, which are TAT5 group D cells complemented with the ATDC cDNA in an expression vector, show a 2.4 kb ATDC mRNA signal and have a nearly normal radioresistance.

Previous reports showed that potentially important functional regions of the ATDC gene are located between exons 1 and 3. These exons contain a leucine zipper motif, a zinc finger-like motif (Leonhardt et al., 1994), a potential phosphorylation site and an ATP/GTPase activity site (Brzoska et al., 1995; Laderoute et al., 1996). These motifs are absent in the ATDC transcript found in AT5B1 group D cells because the splicing alteration in this transcript has resulted in a stop codon being introduced 33 bp downstream from the first methionine codon and the next methionine codon is present in the middle of exon 3.

Western blot analysis showed that two cell lines with normal radioresistance, transformed GM637 cells and diploid HS27 cells, exhibited the presence of full-length ATDC protein (Figure 3). However, no ATDC protein was detectable in diploid AT5B1 cells or in the transformed TAT5 group D cells. These results suggest that the 2.4 kb ATDC transcript in AT group D cells is not translated due to the long 5'-untranslated region with its multiple stop codons which has been introduced into the transcript by the splicing variation. If a peptide is translated, it would have to begin from the mid-point of exon 3 where the next methionine codon is located and, due to the loss of potentially important functional regions, it is not likely to be functional and may not be stable.

The 2.4 kb transcript from normal diploid HS27 cells which express a 2.4 and a 1.6 kb transcript (Hosoi and Kapp, 1994) was also cloned. Some of the 2.4 kb transcripts from these normal cells were sequenced and found to lack exon 1, which was deleted and replaced with a part of intron 1 as a result of an alternative splicing pattern. In these cells, however, the
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Fig. 1. Structure of the cloned ATDC transcripts. The top transcript represents the full-length 3.0 kb ATDC transcript and the bottom shows the AT5BIV A cell ATDC 2.4 kb transcript. The center bar shows the genomic structure of the ATDC gene. The solid arrow indicates a potential start codon in exon 1 at position 125 in the 3.0 kb transcript (top) and a potential start codon (583) in exon 3 in the short 2.4 kb transcript in AT5BIV A cells. In AT5BIV A cells, the 2.4 kb transcript has a part of intron 1 spliced into exon 1 downstream from the ATG codon at bp 125, introducing a series of stop codons (hatched bars) beginning 33 bp past the ATG codon. The next potential start codon in this transcript is located at position 583 in exon 3.

Discussion

The ATDC gene was cloned using functional complementation to identify DNA sequences which could complement the radiosensitivity of AT group D cells in culture. Genomic DNA clones of ATDC provided such complementation and originated from 11Q23, although the original clones were truncated (Kapp and Painter, 1989; Kapp et al., 1992). This suggests that the first 5′-half of the ATDC gene contains most of the activity of the protein. This portion of the gene contains the putative leucine zipper and zinc finger motifs identifying it as a member of an oncogene family (Kakizuka et al., 1991; Kastner et al., 1992; Leonhardt et al., 1994).

Fig. 2. Northern blot analysis of normal and AT group D cells. Northern blot analysis of mRNA from four cell lines is shown. Blots were probed with a full-length ATDC probe. HS27 cells are normal diploid fibroblasts; AT5BI are diploid AT group D fibroblasts; ATDC9 cells are AT5BIV A-transformed cells complemented with ATDC in an expression vector and with near normal radiosensitivity; TAT5 cells (AT5BIV A) are transformed AT5BI cells. The bottom blot shows the results of a β-actin probe used as a control.

Fig. 3. Western blot analysis of AT and normal cells. Extracts from two normal cell lines (GM637 and HS27) and two AT cell lines (diploid AT5BI and its transformant TAT5) were examined for the presence of ATDC protein using an ATDC antibody. The presence of ATDC bands can be seen in the normal cells (GM637 and HS27) but not in the group D AT cells (AT5BI and TAT5).
The findings reported here show that the ATDC protein is non-functional or absent in this AT cell line.

Since nine transcripts of ATDC have been detected in various cell lines and not all normal cells express the 3.0 kb ATDC transcript, it appears likely that other ATDC transcripts can be sufficient for proper cellular functioning. In normal LM217 cells the 1.6 kb ATDC transcript is seen as well as a possibly non-functional 2.4 kp transcript (Hosoi and Kapp, 1994), suggesting that the 2.4 kb ATDC transcript may not be necessary for a normal radiation response. In these cells the 1.6 kb ATDC transcript may code for a functional ATDC protein.

The ATM gene has been found to be mutated in all AT complementation groups, although mutations in both alleles have not always been found (Savitsky et al., 1995; Wright et al., 1996; Stankovic et al., 1998). To date, only one mutated allele of ATM has been detected in the group D cells used here (Savitsky et al., 1995; Wright et al., 1996), leaving the relationship between ATM and AT complementation group D unclear. The ATM gene has also been found to have single allele mutations in several normal cell lines used as controls (Wright et al., 1996), showing that a single allele mutation in ATM is not sufficient to produce radiosensitivity. In AT5BIVA group D cells, ATM has a single allele mutation leading to a truncation of the protein (Savitsky et al., 1995; Wright et al., 1996). Accordingly, these AT5BIVA cells should have a normal phenotype with respect to radiation sensitivity and RDS. If these results are correct, the fact that they are AT-like and possibly have one normal ATM allele suggests that other factors could be involved in producing the AT state in these cells. A functional ATDC protein is not expressed in these cells, but the ATDC protein only complements survival and not RDS, which argues that it also could not be the only gene responsible for the AT state in these cells. However, if the ATM gene is mutated in only one allele in these cells, then the role of ATDC could be of importance in understanding the AT status of AT5BIVA cells.

Alternatively, although a mutation of only one allele of ATM has been found in the AT group D cells used here, the possibility remains that these AT group D cells contain another mutation in the genomic sequence of the other ATM allele or in a non-translated region such as its promoter. These could be difficult to locate due to the large size of the ATM gene (Savitsky et al., 1995).

Assuming that the ATM gene is defective in both AT5BIVA alleles, one model to explain the role of the ATDC gene and protein in AT group D would require that the ATDC gene is necessary for radiation resistance only when the ATM protein has an AT group D type mutation. Since the relationship between ATM mutations and the AT complementation groups is still unclear, an analysis of the ATDC transcript and function could be helpful in identifying the AT group D type mutation in the ATM gene. Although there is a preponderance of null mutations in ATM (Gilad et al., 1996), some AT patients have full-length ATM proteins with a small internal deletion or amino acid substitution (Stankovic et al., 1998). These reports suggest that there might be other cellular proteins whose interaction with ATM might be necessary for normal cellular function. Since the ATDC gene complements radiation sensitivity in AT group D cells, it can be considered a candidate for this role.

An understanding of the role of the ATDC gene in AT group D cells, as well as of ATM gene expression in these cells and possibly of the involvement of other genes, yet to be identified, may be required to fully understand and explain AT.

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References

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Note added in proof
While this paper was in press, Combining Serial Analysis of Gene Expression and Array Technologies to Identify Genes Differentially Expressed in Breast Cancer, by Nacht et al. (Cancer Res., 59, 5464–5470, 1999) was published. This paper looked at differential gene expression by comparing gene expression in breast tumor cells with expression in normal mammary epithelial cells. Differential expression was observed in a group of genes, and among these the ATDC gene was shown to be expressed at greatly reduced levels in breast tumor cells when compared to expression in normal mammary epithelial cells. This observation is of interest due to the suggested association of breast cancer with AT heterozygotes.