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Transposon Tc1 of the nematode *Caenorhabditis elegans* jumps in human cells

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ABSTRACT

The transposon Tc1 of the nematode *Caenorhabditis elegans* is a member of the widespread family of Tc1/mariner transposons. The distribution pattern of virtually identical transposons among insect species that diverged 200 million years ago suggested horizontal transfer of the elements between species. This hypothesis gained experimental support when it was shown that Tc1 and later also mariner transposons could be made to jump *in vitro*, with their transposase as the only protein required. Later it was shown that mariner transposons from one fruit fly species can jump in other fruit fly species and in a protozoan and, recently, that a Tc1-like transposon from the nematode jumps in fish cells and that a fish Tc1-like transposon jumps in human cells. Here we show that the Tc1 element from the nematode jumps in human cells. This provides further support for the horizontal spread hypothesis. Furthermore, it suggests that Tc1 can be used as vehicle for DNA integration in human gene therapy.

INTRODUCTION

The Tc1 transposon of the nematode *Caenorhabditis elegans* is the prototype of the Tc1/mariner family of transposable elements. Members of this family have been found in the genomes of nematodes, arthropods and vertebrates; even the human genome contains Tc1-like elements (1–6). The successful spread of this type of transposon could be explained by the presence of a Tc1-like element in the common ancestor of these species or via, relatively recent, horizontal transfer between species. This latter hypothesis gained support when it was shown that insect species that diverged more than 200 million years ago contain almost identical Tc1-like elements (7). Further support came when it was shown that the only protein required to carry out the transposition reaction *in vitro* is the transposase protein encoded by the element itself; no host-specific factors are required (8,9). This is in contrast to some insect transposons (such as the P element) that seem unable to transpose in unrelated animal species due to the requirement for transposition of a host-encoded protein (10). Recent *in vivo* experiments show that Tc1-like elements can jump in heterologous species. It was shown that mariner transposons from one fruit fly species can jump in other fruit fly species (11,12), that a mariner element from insects can jump in the protozoan *Leishmania* (13), that a Tc1-like transposon from fish transposes in human cells (14) and a Tc1-like element from *C.elegans* can jump in fish cells (15). Here we report that the nematode Tc1 element can jump in human cells.

Tc1/mariner transposons consist of inverted repeats that flank a single gene encoding the transposase. Most work has been done on the Tc1 and Tc3 elements, but it seems reasonable to assume that the mechanistic insights obtained for Tc1 apply equally to all other Tc1/mariner transposons. In short, the N-terminus of the transposase specifically recognizes the terminal part of the transposon inverted repeats (8,16,17). Once bound to the termini of an integrated transposon, the transposase excises the element by double-strand breaks. The complete transposon sequence is not excised: the two most 5′ nucleotides on both sides of the transposon are not part of the excised transposon but remain attached to the donor site (18). The double-strand break at the empty donor site may be repaired by the cellular repair machinery in different ways, often resulting in characteristic footprints (18,19). Tc1 and Tc1-like elements always integrate into the sequence TA. Since integration occurs by nucleophilic attacks on phosphodiester bonds that are not directly opposite in the target DNA, the end result of integration (after repair of single-strand regions and nicks) is a duplication of the TA sequence.

The *cis*-requirements for Tc1 and Tc3 jumping are limited to <100 terminal base pairs (8; H.G.A.M.van Luenen and R.H.A.Plasterk, unpublished observations). The *trans*-requirements are similarly simple: the transposase protein alone can carry out the reaction in a simple buffer without co-factors (8). Based on the simple *cis*- and *trans*-requirements for Tc1 jumping, several studies were initiated to test the capacity of Tc1 and Tc3 to jump in different hosts. Here we show that the nematode transposon Tc1 jumps into the human genome.

MATERIALS AND METHODS

Construction of plasmids

The plasmid pTc1.neo-TK was constructed as follows. pRP466 (8) was digested with *Xho*I and blunted with Klenow and dNTPs. The plasmid pRC/CMV (Invitrogen) was digested with *Bam*HI

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and *Eco*RI and blunted with Klenow and dNTPs. Subsequently, the *Bam*HI–*Eco*RI SV-*neo* fragment was cloned into *Xho*I-digested pRP466, giving rise to pRP466.neo. The plasmid pRP466.neo was digested with *Eco*RI and blunted with Klenow and dNTPs. The plasmid pPGK-TK (PCT/NL/00195) was digested with *Pvu*II. The *Pvu*II PGK–*TK* fragment was cloned into *Eco*RI-digested pRP466.neo, giving rise to pTc1.neo-TK.

The plasmids pRC/CMV.Tc1A and pcDNA1.Tc1A contain the Tc1 transposase cDNA, which was amplified from pRP470 (8) with the primers 5′-CCCCAAGCTTGCCACCATGGTAAAAT-CTGTTGGGTGTAAAAATC and 5′-GCTCTAGATGCTTAA-TACTTTGTCGCGTATCC using eLONGase according to the standard protocol of the supplier (Gibco BRL). PCR was performed on a Biometra Trio Thermoblock, amplification program: 94°C for 1 min, 1 cycle; 94°C for 30 s, 52°C for 30 s and 68°C for 1 min, 35 cycles; 68°C for 1 min. The PCR fragment was digested with *Hin*dIII and *Xba*I and cloned into *Hin*dIII/*Xba*I-digested pRC/CMV or pcDNA1 (Invitrogen; the pRC/CMV vector carries the neomycin resistance marker). The integrity of the amplified *Tc1A* gene was confirmed by sequencing. All chemicals, enzymes and primers were purchased from Gibco BRL.

Cell culture and transfections

The cell line 911 (20) was cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS at 37°C and 5% CO_2. Selection of transfected cells with G418 was performed at a concentration of 500 µg/ml. Selection with Ganciclovir (Roche) was performed at a concentration of 10 µg/ml. DNA was transfected into 911 cells using the calcium phosphate transfection system, according to the manufacturer's protocol. Briefly, 1 day prior to transfection, 911 cells were plated at 30% density on 5 cm tissue culture dishes (Greiner). The next day, cells were incubated with calcium phosphate-precipitated DNA for 15 h. Subsequently, the cells were washed twice with PBS and fresh medium was added. Approximately 48 h post-transfection cells were put on G418 selection medium. G418-resistant colonies appeared around day 6. Colonies were stained with methylene blue. Single colonies were picked and cell lines were established.

Protein detection

Western blotting was performed as described previously (21). Briefly, cells were washed twice with PBS (NPBI) and lysed and scraped into RIPA (1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS in PBS, supplemented with 1 mM phenylmethylsulfonyl fluoride and 0.1 mg/ml trypsin inhibitor). After 15 min incubation on ice, the lysates were cleared by centrifugation. Protein concentrations were determined by the BioRad protein assay, fractionated by SDS–PAGE on 10% gels. Proteins were transferred onto Immobilon-P membranes (Millipore) and incubated with an anti-Tc1 transposase antibody (22). The secondary antibody was a horseradish peroxidase-conjugated goat anti-rabbit antibody (BioRad). The antibody complexes were visualized with the ECL detection system according to the manufacturer's protocol.

Cell fractionation

Transfected cells were washed twice with PBS (NPBI). Cells were scraped, spun down and resuspended in NIB (25 mM Tris, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 5 mM DTT and 0.1 mM phenylmethylsulfonyl fluoride). After 10 min incubation on ice, 10 µl 10% Tween-20 was added. The mixture was shortly vortexed, incubated on ice for 1 min and briefly spun down. The supernatant was collected (cytoplasmic fraction). The pellet was resuspended in NEB (25 mM Tris, pH 7.5, 0.1 mM EDTA, 50 mM NaCl, 15% glycerol, 0.25% Tween-20, 1 mM DTT and 0.1 mM phenylmethylsulfonyl fluoride). The lysate was incubated on ice for 15 min and cleared by centrifugation.

Detection of transposition

In vitro excision reactions have been performed as described in Vos *et al*. (8). The products of the *in vitro* excision reaction were separated on a 0.8% agarose gel in 0.5× TBE by electrophoresis for 5 h at 70 V. Subsequently the gel was rinsed in Southern blot buffer (0.4 N NaOH, 0.6 M NaCl) for 30 min and the DNA was blotted overnight onto Hybond-N^+ (Amersham). After blotting, the blot was rinsed in 5× SSPE and pre-hybridized for 3 h in 50% formamide, 5× Denhardts, 5× SSPE, 5% dextran sulphate, 1% SDS and 200 µg/ml herring sperm DNA and overnight hybridized with a randomly primed ^{32}P-labelled (RTS Radprime DNA labelling system; Gibco BRL) *neo* probe (*Bam*HI–*Eco*RI fragment of pRc/CMV) at 42°C. Subsequently the blot was extensively washed in 5× SSPE, 0.1% SDS at 65°C. The blot was exposed to HyperFilm (Amersham) at room temperature.

Transposon flanks were visualized using a PCR-based display method (23). Genomic DNA (100 ng) was digested with 4 U *Sau*3A restriction enzyme for several hours, after which the restriction enzyme was heat inactivated. Equimolar amounts of the two vectorette oligos (503, 5′-GATCCAAGGAGAGGAC-GCTGTCTGTCGAAGGTAAGGAACGGACGAGAGAAGGG-AGA; 504, 5′-TCTCCCTTCTCGAATCGTAACCGTTCGTA-CGAGAATCGCTGTCCTCTCCTTG) were annealed in TE. An aliquot of 15 pmol annealed vectorette cassette was ligated overnight at 16°C to the digested genomic DNA in a 100 µl volume using 5 U T4 DNA ligase (Boehringer Mannheim). An aliquot of 3 µl ligated DNA was used in a first PCR with 10 pmol vectorette-specific primer (505, 5′-CGAATCGTAACCGTTCG-TACGAGAATCGCT) and 10 pmol transposon-specific primer (Tc1L1, 5′-TGTTCGAAGCCAGCTTACAATGGC for the left flank of Tc1 or Tc1R1; 5′-GCTGATCGACTCGAGCCACGTCG for the right flank of Tc1) in a final volume of 25 µl using 1 U *Taq* DNA polymerase (Gibco BRL). Twenty cycles were performed with 1 min at 95°C, 1 min at 58°C and 1 min at 72°C. The product of the first PCR was diluted 100 times and 1 µl was used to start a second PCR using 10 pmol nested vectorette primer (337NEW, 5′-GTACGAGAATCGCTGTCCTC) and 1 pmol radiolabelled nested transposon-specific primer (Tc1L2, 5′-TCAAGTCAAA-TGGATGCTTGAG, or Tc1R2, 5′-GATTTTGTGAACACTGT-GGTGAAG) using the same PCR conditions. The PCR products were separated on a 6% denaturing polyacrylamide gel. After electrophoresis the gel was dried onto GB002 blotting paper (Schleicher and Scheull), fluorescent markers were placed on the gel and the gel was exposed to X-OMAT film (Kodak).

To determine the insertion site bands were cut out of the paper by aligning the film to the paper. The slices of dried gel (attached to the paper) were put in 10 µl water and heated to 65°C for at least 10 min. A sample of 1 µl eluate was used for a PCR with 10 pmol nested vectorette and transposon primers; the PCR conditions were the same as described above except that the PCR continued

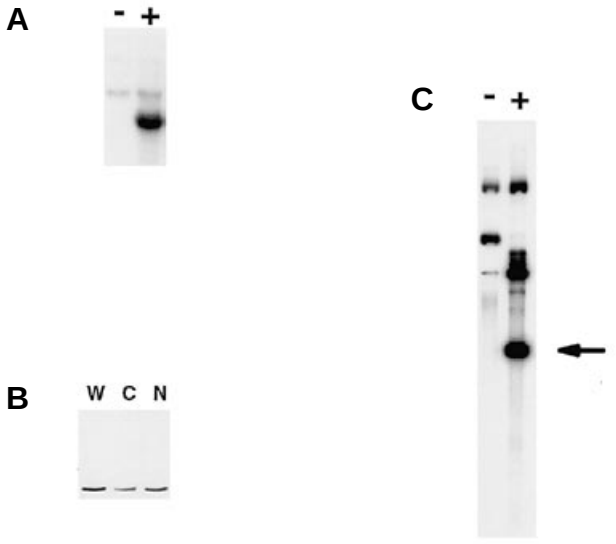

Figure 1. Tc1 transposase expression in human cells. (**A**) Western blot showing expression of the Tc1 transposase protein after transfection of the human retina 911 cell line with pRC/CMV.Tc1A (lane marked +). The lane marked – contains a cell lysate from the 911 cell line transfected with the empty pRC/CMV vector. (**B**) 911 cells expressing Tc1 transposase were fractionated to determine the cellular localization of the protein by western blot analysis. W, whole cell lysate; C, cytoplasmic extract; N, nuclear extract. (**C**) The artificial Tc1.neo transposon can be excised from its vector using purified recombinant transposase from *E.coli*. The plasmid containing the neomycin marked Tc1 element was incubated with (+) or without (–) Tc1 transposase as described in Vos *et al.* (8). After incubation the DNA was isolated and analysed on a Southern blot using a radiolabelled *neo* probe. The arrow indicates the position of the excised Tc1 elements.

for 30 cycles. The amplified fragments were purified (QIAEX II; Qiagen) and cloned into the pGEM-T vector (Promega) and sequenced using a transposon-specific primer.

RESULTS

The Tc1 transposase gene (*Tc1A*) was put under control of the CMV promoter and transfected into 911 cells, a human embryo retina cell line immortalized by early region I of adenovirus type 5 (20). As shown in Figure 1A, we could detect Tc1A protein, confirming expression of the transposase gene. The Tc1A protein is present both in the cytoplasm and the nucleus of the 911 cells (Fig. 1B). We constructed a Tc1 element carrying the *neomycin* resistance gene in between the terminal repeats required in *cis* for jumping and a *TK* expression cassette outside the transposon. This artificial transposon could be excised from its vector in an *in vitro* excision assay. After incubation of the plasmid with purified Tc1A from *Escherichia coli* we could detect an excised product of the predicted lenght (indicated by an arrow in Fig. 1C), showing that the artificial Tc1 transposon is a substrate for Tc1A.

Subsequently, the plasmids pcDNA1.Tc1A and pTc1.neo-TK were co-transfected into the 911 cell line. As shown in Table 1, we reproducibly found a significantly higher number of G418-resistant colonies when transposase was co-transfected (an ~50% increase; $P = 0.09$ in a paired samples *t*-test). This suggests that the transposase protein has stimulated integration of the antibiotic marked transposon. The G418-resistant colonies in the negative control experiments (when no transposase was present) are probably the result of transposase independent, non-homologous recombination. To test this we established independent G418-resistant cell lines by co-transfection of 0.1 µg pTc1.neo-TK and either 2 µg pcDNA1.Tc1A or 2 µg pcDNA1. When transposase was not present, 68% (13 of 19) of the G418-resistant colonies were Ganciclovir-sensitive, whereas only 23% of the G418-resistant colonies were Ganciclovir-sensitive when transposase was present. This shows that >50% of the G418-resistant colonies after co-transfection with the transposase gene have lost the vector sequence which carries the *TK* expression cassette. Not all G418-resistant colonies transfected without transposase have an active *TK* gene. This is probably due to non-homologous recombination events disrupting the *TK* gene. Any colonies derived from a cell where there was integration by non-homologous recombination in addition to transposition should be sensitive to Ganciclovir and thus could be discarded. The frequency of integrations might therefore be higher than indicated.

We investigated at the molecular level whether transposase-mediated transposition had occurred. A genuine transposition event would imply precise fusion of the Tc1 terminus to a target sequence which starts with the sequence TA. DNA from five independent G418-resistant, Ganciclovir-sensitive colonies were isolated and digested. A oligo vectorette was ligated to the fragments (23). Fragments carrying a transposon junction were specifically amplified by PCR using an oligo in the transposon and one in the vectorette. A second PCR was performed using nested oligos; one of the oligos (the oligo located in the transposon) being radiolabelled at the 5′-end. The fragments were analysed on a polyacrylamide gel (Fig. 2). Each lane contains one or more bands, suggesting one or multiple insertions of the transposon. The weaker bands could be the result of secondary transposition events in a sub-population of the colony forming cells. This would be possible when the transposase expression plasmid had integrated in the 911 genome.

Table 1. Co-transfection of a neomycin gene marked Tc1 element and a Tc1 transposase expression construct in a human retina cell line

Plasmid	G418-resistant colonies per plate			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
0.1 µg pTc1.neo-TK + 2 µg pcDNA1.Tc1A	70	54		
0.1 µg pTc1.neo-TK + 2 µg pcDNA1	38	30		
0.5 µg pTc1.neo-TK + 2 µg pcDNA1.Tc1A	205	223		
0.5 µg pTc1.neo-TK + 2 µg pcDNA1	150	140		
0.1 µg pTc1.neo-TK + 1 µg pcDNA1.Tc1A[a]	177	202	211	252
0.1 µg pTc1.neo-TK + 1 µg pcDNA1[a]	113	121	177	138

[a]Transfection was performed using Lipofectamine instead of $CaPO_4$.

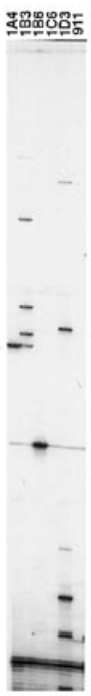

Figure 2. Display of fragments containing Tc1 termini and flanking sequences. DNA from five independent G418-resistant colonies co-transfected with pcDNA1.Tc1A and pTc1.neo-TK and the parental 911 cell line was isolated and digested with *Sau*3A. To these fragments a double-stranded oligonucleotide was ligated. The right Tc1 flanks were amplified by PCR using a primer in the Tc1 right terminus and a primer in the cassette that was ligated to the restriction fragments. To increase the sensitivity of this assay, a second PCR is performed with nested primers (the nested transposon primer was radiolabelled). Fragments were analysed on a sequencing gel. The display shows only the right flanks of the Tc1 transposon.

To confirm that these bands represent real transposition events, we isolated fragments from the gel and determined the sequence. Most of the Tc1 termini were still flanked by vector sequences, but we also recovered Tc1 termini flanked by new sequences (Fig. 3). All these flanks contained the terminal Tc1 sequence, followed by TA and a new sequence. This shows that *bona fide* jumping of Tc1 had occurred in these human cells.

DISCUSSION

This paper shows for the first time that the nematode Tc1 transposon can jump in human cells. It had been shown before that Tc1/mariner elements can jump in other species than the ones in which they were discovered: insect mariner elements jump in other related insect species (11,12), but also jump into the protozoan kingdom (13), and the nematode Tc3 element jumps in fish (15). Here we show that the nematode Tc1 element can make an even further jump into the human genome. While this work was in progress, an independent line of research led to the synthesis of an active transposase from a series of mutant pseudogenes found in the salmon genome; the result of this molecular archaeology was termed Sleeping Beauty (14). The Sleeping Beauty element can also jump in human cells. To test whether there are differences in the frequency of jumping for the different Tc1/mariner elements, we are currently performing experiments to compare several elements for their transposition frequency in human cells using similar constructs and identical conditions.

The applications of Tc1 jumping in human (and other mammalian) cells are several. First, gene tagging in mice has proven to be a powerful method to identify oncogenes in mammary tumours and lymphomas (24), an approach that has thus far been limited to tissues that can be reached by retroviruses (MMTV and MLV respectively). Given the possibility of expressing Tc1 transposase specifically in one tissue, it may be possible to use somatic gene tagging with Tc1 to identify oncogenes involved in several other tumours, such as colon, brain, etc. Secondly, gene tagging in the germline of the mouse has been tried on a very small scale (25), but has not been found to be an efficient method of identifying genes. It remains to be seen whether the frequency of Tc1 jumping in the germline of the mouse is high enough to allow for successful gene tagging. If so, an important bottleneck in forward genetics of the mouse would be solved. Finally, Tc1 can be used to develop gene therapy vectors. The currently used vectors are either not efficient in delivering genetic material into post-mitotic cells (such as retroviruses) or they do not stably integrate the DNA into the genome of the targeted cells (e.g. adenoviruses; 26). A solution may be to use a vector that is an efficient and safe delivery vehicle (an adenovirus or a non-viral method) and use those to deliver DNA that contains a transposon that can integrate the relevant

pTc1.neo-TK: GAGACGAG**TA**cagtg.....Tc1.neo.....cactg**TA**TTTCTATG

flanks from G418-resistant human cell lines:

vector flanks:
cactg**TA**TTTCTATG
cactg**TA**CTCGTCTC

new flanks:
cactg**TA**CATGCAACGATAATCATTCCGAAAAACGCCATAATCCCCA(1B3)
cactg**TA**GATTTTCCCCGGTGGGAGCAAGAATTNTTNAAAGATTACG(1B6)
cactg**TA**ACTGTGTTGCAGCAGTTCTGGACTCACGATCTTTCCAGCC(1C6)
cactg**TA**TTAGGAACTCATTTACTTCCCGTTNTGTGAAGAACACTGT(1D3)

Figure 3. Tc1 and its flanking sequence from the vector pTc1.neo-TK and from DNA isolated from G418-resistant colonies transfected with a neomycin gene marked Tc1 element (pTc1.neo-TK) and a Tc1 transposase expression construct (pcDNA1.Tc1A). The TA sequences are indicated in bold, flanking sequences are indicated in capitals and the cell line from which the flank has been isolated is indicated between brackets. The junction between the transposon and the genomic sequence is precisely at the transposon end.

part of the vector into the genome. If the transposase gene is not contained within the transposable element, then the final effect of the gene therapy may be a stably integrated transgene.

Several questions still need to be addressed and are the subject of on-going research. As mentioned above, it is important to test the relative frequencies of transposition into human or rodent cells of any of the Tc1/mariner family members known to jump. In addition to that, one could consider genetic screens aimed at isolation of mutant derivatives of those transposases that are more active.

It will also be important to determine to what extent the size of the transposon reduces the transposition frequency. We have indications that inserts of up to 15 kb do not greatly reduce the frequency of jumping of Tc3 *in vivo* and *in vitro* (H.G.A.M.van Luenen and R.H.A.Plasterk, unpublished observation).

For gene tagging purposes, the target choice is important. As discussed elsewhere (27), it is likely that some genes are hit more often than others. However, based on the experience in *C.elegans*, we expect the large majority of genes to be good targets for Tc1 and its relatives.

In conclusion, this study shows that Tc1 and its relatives might become important tools in mammalian genetics and gene therapy.

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