Nuclear targeting of a bacterial integrase which mediates site-specific recombination between bacterial and human target sequences

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Abstract

TrwC is a bacterial protein involved in conjugative transfer of plasmid R388. It is transferred together with the DNA strand into the recipient bacterial cell, where it can integrate the conjugatively transferred DNA strand into its target sequence present in the recipient cell. Considering that bacterial conjugation can occur between bacteria and eukaryotic cells, this protein has great biotechnological potential as a site-specific integrase. We have searched for possible TrwC target sequences in the human genome. Recombination assays showed that TrwC efficiently catalyzes recombination between its natural target sequence and a discrete number of sequences, located in non-coding sites of the human genome, which resemble this target. We have determined the cellular localization of TrwC and derivatives in human cells by immunofluorescence, and also an indirect yeast-based assay to detect both nuclear import and export signals. The results indicate that the recombinase domain of TrwC (N600) has nuclear localization, but full-length TrwC locates in the cytoplasm, apparently due to the presence of a nuclear export signal in its C-terminal domain. The recombinase domain of TrwC can be transported to recipient cells by conjugation in the presence of the helicase domain of TrwC, but with very low efficiency. We mutagenized the *trwC* gene and selected for mutants with nuclear localization. We obtained one such mutant with a point A904T mutation and an extra peptide at its C-terminus, which maintained its functionality in conjugation and recombination. This TrwC mutant could be useful for future TrwC-mediated site-specific integration assays in mammalian cells.

Introduction

Bacterial conjugation is a specialised mechanism to transfer plasmid DNA from a donor to a recipient bacterial cell. Under laboratory conditions, conjugative DNA transfer has been described to occur from bacteria to *S. cerevisiae* (19), plants (3) and mammalian cells (45). The molecular process is related to the naturally occurring inter-kingdom gene transfer process from *Agrobacterium tumefaciens* to plant cells (47).

Mechanistically, bacterial conjugation has been described as a two step process involving conjugative DNA processing followed by active DNA transport (30). DNA processing is driven by a nucleoprotein complex called relaxosome. Within it, a relaxase protein introduces a site and strand specific nick into the origin of transfer (*oriT*), remaining convalently bond to the 5' end of the DNA strand to be transferred. For the DNA transport, a set of proteins constitute a type IV secretion system (T4SS) that forms a transmembranal channel. Transfer of the DNA molecule is proposed to occur in two steps: i) the secretion of the relaxase protein (with the attached DNA strand) through the T4SS, and ii) pumping out of the remaining DNA molecule by a specialised ATPase (30). Thus, the relaxase also functions as a pilot protein guiding the DNA molecule into the recipient cell, where it presumably acts recircularizing the transferred DNA strand (10).

TrwC is the relaxase of the conjugative system of plasmid R388 (31). Its 966 amino acids comprise several functional domains, as shown in **Fig. 1a**. The crystal structure of the N-terminal relaxase domain has been determined (17). It shares a central structural domain with the HUH family of proteins, which include the rolling circle replication initiation proteins (5) and the Rep protein of adeno-associated virus (AAV) (21), which catalyses the integration of the viral genome into a unique site in the human genome (41). The C-terminal domain shows oligomerization and DNA helicase activities. Both the relaxase and helicase domains

expressed in the same cell reconstitute TrwC function in conjugation to a certain extent (32).

TrwC is also a site-specific recombinase capable of promoting efficient recombination between two cognate *oriT*s on a double-stranded (ds) DNA molecule (28). The relaxosomal component protein TrwA is required for high efficiency recombination (7). The minimal recognition sequences for efficient TrwC-mediated recombination show different requirements at each *oriT* locus: a core 17 bp sequence is sufficient to host efficient recombination in locus 1, while an additional 183 bp sequence is required at locus 2 (7), with the *nic* site located 3´ to locus 1. The recombination reaction is strictly dependent on the nicking and strand-transferase abilities of TrwC, since a mutation in the two catalytic tyrosine residues completely abolished recombination (10). However, the relaxase domain alone was not sufficient to catalyze recombination; the recombinase domain has been assigned to the N-terminal 600 residues of TrwC (7).

Fig. 1b shows the R388 *oriT* sequence requirements for different TrwC activities. The common 17 bp core sequence comprises a highly specific sequence termed the *nic* site, where the scissile phosphate lies, and a 5' region involving the recognition hairpin formed by an inverted repeat, IR_2 (17). This is the essential core sequence required *in vivo* for both conjugal mobilization (33) and site-specific recombination (7). When the distal half of IR_2 is removed, TrwC binding affinity is decreased only three fold and TrwC-mediated *in vivo* site-specific recombination is slightly affected (7). In contrast, the sequence 6+2 is absolutely required for TrwC binding, single-strand nicking and strand transfer reactions (33). Similar results were reported for the related TraI relaxase of the F plasmid (46), where mutations affecting the nucleotides immediately adjacent to the *nic* site had a strong detrimental effect on conjugation.

Once TrwC enters the recipient cell it is fully active, as shown by complementation of a *trwC*-deficient mutant in the recipient (10). Notably, TrwC can also catalyze integration of a conjugatively transferred *oriT*-containing molecule

into a recipient dsDNA *oriT* target (10). To our knowledge, this ability of TrwC to act as a site-specific integrase is unique amongst relaxases; even the related F TraI protein cannot catalyze a similar reaction (7). Protein VirD2 of *Agrobacterium tumefaciens*, functionally related to conjugative relaxases, has been postulated to reach the recipient plant cell, where it directs nuclear import of the transferred DNA and aids T-DNA integration into the plant genome, preserving the integrity of the T-DNA 5´end (34). However, this integration is not site-specific. In addition to VirD2, conjugative relaxases TraI and MobA from plasmids RP4 and RSF1010, respectively, have been localised within the nucleus of human cultured cells (40). However, integration assays in plants showed a specific deficiency of MobA in aiding genomic integration of the transferred DNA (2).

Given the observed capacity for bacterial conjugation to transmit DNA to mammalian cells, this feature of TrwC as a site-specific integrase upon entering the recipient cell suggests a potential biotechnological use of TrwC as an engineering integrase delivered *in vivo* by conjugation together with the transferred DNA (29). There are many prokaryotic recombinases used for genetic manipulation in mammals, but for most of them their target cells need to be modified in advance by addition of the recombinase target sequence to their genomes (42). In this work, we show that TrwC catalyzes recombination between its cognate *oriT* and human sequences closely resembling the *nic* site. We also address TrwC nuclear targeting, since a recombinase must target the nucleus in order to work efficiently in eukaryotic cells. By random mutagenesis we obtain a full-length TrwC mutant targeting the nucleus which is fully functional in conjugation and recombination.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions. *E.coli* strain DH5α (16) was used as a host for the recombination assays. For mating assays, strains D1210 (38) and DH5α were used as donors and recipients, respectively. Plasmids used in this work are listed in **Tables 1** and **2**. Luria-Bertani broth was used for bacterial growth, supplemented with agar for solid culture. Selective media included antibiotics at the following concentrations: ampicillin (Ap), 100 μg/ml; chloramphenicol (Cm), 25 μg/ml; kanamycin (Km), 25 μg/ml; nalidixic acid (Nx), 20 μg/ml; streptomycin (Sm), 300 μg/ml; trimethoprim (Tp), 20 μg/ml. X-Gal was supplied at a concentration of 60 μg/ml.

Plasmid constructions. Plasmids were constructed using standard methodological techniques (39). Restriction enzymes, Shrimp Alkaline Phosphatase, T4 DNA ligase and T4 polynucleotide kinase were purchased from Fermentas. Vent polymerase was purchased from New England Biolabs. DNA sequences of all cloned PCR segments were determined. Oligonucleotides used in the amplification of human sequences were designed using the Primer3:WWW primer tool (http://biotools.umassmed.edu/bioapps/primer3_www.cgi (37)) setting an annealing temperature of 60 °C and 400 bp product size. Oligonucleotide hybridisation was performed as described (7).

Table 2 includes the details of how each plasmid was constructed. A brief description of each set of plasmids follows.

- All substrate plasmids for recombination assays are derivatives of plasmid pCIG1028 (7), which contains two wild-type *oriT* copies separated by a kanamycin-resistance gene and a *lacI*^{Q} repressor gene in vector pSU19. The 1-402 bp wild-type sequence of *oriT1* copy was substituted by the indicated mutations or human sequences. The latter were amplified from human genomic DNA obtained from a K562 leukemia cell line (ATCC).

- For intragenic relaxase-helicase complementation, different *trwC* 5´ terminal fragments were cloned in vector pSU19 under the control of the lactose promoter. All constructs carry the start codon at the same position with respect to the transcription/translation signals of the vector.

- Constructs to test nuclear import/export in yeast were made on vectors pNIA3b/pNEA3b (36), which carry appropriate sites to make protein fusions with LexA-Gal4AD and LexA- nuclear localization signal (NLS)-Gal4AD, respectively. pCMS5 carries a NLS at the C-terminus of TrwC; the oligonucleotide used to amplify *trwC* added a sequence coding for the peptide GKKGR just before the stop codon. To construct NLS-TrwC derivatives, oligonucleotides including the coding sequence for the SV40 NLS were hybridized and 5' phosphorylated with T4 polynucleotide kinase. Resulting fragment was cloned into the BamHI site of plasmid pCMS9 (pNIA:*trwC*). Two constructs were selected: pMTX719 contains five inserts that coded for NLS sequences in direct orientation and a last one in the opposite orientation, without affecting the *trwC* reading frame; pMTX720 carries 3 copies of the insert, two of them in the sense orientation, and the middle one in opposite orientation. To obtain a construct with a single NLS fused to the N-terminus of TrwC, pMTX719 was digested with BamHI and religated, obtaining pMTX726. Construction of pLA44 is detailed in the "Random mutagenesis" section below. Plasmid pLA66 was constructed from pLA44 as indicated in Table 2 to separate mutations present in pLA44.

- For immunofluorescence analysis, *trwC* and derivatives were subcloned into the pCEFL expression vector (43) and expressed from the eukaryotic promoter elongation factor 1α (EF1α).

TrwC binding assays. Binding of TrwC-N293 to oligonucleotides was determined by an electrophoresis mobility shift assay as previously described (17). Oligonucleotides used were either R388 (25+8) or those containing the homologous human sequences shown in **Fig. 2a**. The 25+8 nomenclature refers to the 25

nucleotides 5′ and 8 nucleotides 3′ to the *nic* site. 1 nM 5′-labelled (25+8) oligonucleotides were incubated with 0-20 nM TrwC-N293 purified as described (17) in the presence of 1 μM competitor oligonucleotide (a mixture of 3 unlabeled unspecific oligonucleotides).

DNA strand transfer assays. Oligonucleotides were 5′-labelled and strand-transfer assays were performed as described (14). Assays contained 250 nM cold "donor" oligonucleotide R388(12+18) 5′- TGCGTATTGTCT/ATAGCCCAGATTTAAGGA -3′ (the slash indicates the R388 *nic* site according to (31)), 50 nM of the indicated "acceptor" (25+8) labelled oligonucleotides and 500 nM TrwC-N293 protein. Gels were scanned on a Molecular Imager FX system and strand-transfer products were quantified using "Quantity One" software (BioRad) and expressed as the percentage of the total label in each lane.

Recombination assays. Intramolecular recombination was tested as described (7). Briefly, the substrate plasmid (pCIG1028 or its derivatives) carries two copies of the R388 *oriT.* Recombination between the two copies induces expression of the downstream *lacZ*α gene. A plasmid coding for TrwAC under the regulation of the *trwA* promoter was used as a helper plasmid. A plasmid coding for TrwA only was used as helper in the negative control. To test the recombination activity of TrwC derivatives in pNIA or pCEFL, plasmid pCIG1030, as pCIG1028 but coding also for *trwA*, was used as a substrate (7). The substrate and helper plasmids were introduced into the *lacZ*Δ*M15* strain DH5α and plated on selective media with X-gal. Recombination activity is estimated by the number and size of blue sectors in bacterial colonies.

Mating assays. Standard mating assays were performed as described (14). *E. coli* D1210 donor cells contained plasmid pSU1445, a TrwC deficient R388 mutant, which was complemented with plasmids coding for TrwC derivatives *in*

trans. For the relaxase-helicase complementation assays, a combination of compatible plasmids coding for C-terminal and N-terminal domains of TrwC was used to complement the transfer deficiency of plasmid pSU1445. Mating assays were performed in the presence or absence of IPTG, and no significant differences were found (data not shown). A plasmid coding for wild-type TrwC was used as a positive control and the corresponding empty vector was assayed as a negative control in all cases.

Immunofluorescence assays*.* Human embryonic kidney 293T cells were maintained in DMEM supplemented with 10% fetal calf serum. $6x10^5$ 293T cells were placed in 10 ml DMEM on glass coverslips and grown for 48h. Cells were transfected with 7 μg DNA + 14 μl JetPei (Genycell). After 24h, cells were fixed and permeabilized with methanol for 10 min at -20 °C, and immunostaining was performed as previously described (44). Cells were successively incubated with primary anti-TrwC antibody (15) (3h incubation at RT) and secondary FITC-conjugated goat anti-rabbit antibody (Jackson Laboratories), both used at a 1:100 dilution. Coverslips were mounted with anti-fading mounting medium Vectashield (Vector Laboratories) with 4',6-diamidino-2-phenylindole (DAPI) to visualize the nucleus. Cell samples were examined using a Zeiss Imager M1 fluorescence microscope.

Nuclear import/export assays. Nuclear import/export assays in yeast were done as described (36). The *Saccharomyces cerevisiae* strain L40 (22) contains *HIS* and *lacZ* genes which are expressed upon binding of the LexA-Gal4AD transcriptional regulators. The pNIA vector codes for a LexA-Gal4AD fusion to which the test protein is fused; if the test protein drives the fusion to the nucleus, then the reporter genes are expressed. L40 cells were transformed by the LiAc-PEG method (12) with the pNIA/pNEA derivatives. Transformants were grown in tryptophan dropout minimal medium (DO-trp) for plasmid selection. Individual

transformants were picked from these plates, grown on liquid DO-trp overnight at 30ºC, and 20 μl of these cultures were streaked on DO-trp plates as positive controls and on histidine dropout minimal medium plates (DO-his) to select for HIS expression. β-galactosidase activity was assayed with X-gal on permeabilized yeast cells transferred to nitrocellulose filters, as described (22).

Random mutagenesis. Random mutagenesis of full-length TrwC was performed to select for TrwC mutants in pCMS9 entering the yeast nucleus (and thus growing in DO-his medium when introduced in yeast strain L40). Mutations were introduced by mutagenic PCR using the GeneMorph II Random Mutagenesis Kit (Pharmacia) following the manufacturer´s recommendations. We adjusted the reactions to obtain 0-3 mutations per kb. Template DNA was pCMS9. Oligonucleotides used were GGCTGGCGGTTGGGGGTTA, annealing in *lexA* towards the beginning of *trwC*, and ACCGAATT**TCA**CCTTCCGGCCTCCATGCCGCG, annealing at the end of *trwC* (complementary sequence to *trwC* stop codon shown in bold, EcoRI site underlined). Full-length *trwC* from the PCR amplified products was obtained by digestion with enzymes BamHI + EcoRI and cloned into the corresponding sites of pCMS10 (replacing the C774 TrwC fragment, which does not enter the yeast nucleus). In order to maximize the transformation efficiency, ligations containing the pool of mutants were first introduced in *E. coli* DH5α, then DNA extracted from the pool of colonies and introduced into the yeast strain in successive transformation events. About four thousands colonies were collected from DH5α, from which 34 μg DNA was extracted and used to transform competent L40 yeast cells, carrying the reporter HIS gene; 1-2μg of DNA was used per L40 aliquot. Transformations were incubated in DO-trp medium for 8 h at 30ºC, and then 1/10th plated on DO-trp plates (to calculate transformation efficiency) and the rest plated on DO-his plates. Plasmid DNA was extracted from His+ colonies using the yeast DNA extraction kit (Thermo Scientific) and introduced in DH5α for further analysis.

Western blots. In order to detect expression of TrwC fusions in yeast, cells were lysed and processed as follows. ~10^8 yeast L40 cells containing the pNIA derivatives were collected by centrifugation and frozen on dry ice. The pellet was then thawed at room temperature and resuspended in 300 µl of 20% Tri-chloro acetic acid (TCA). 300 µl of glass beads (425-600 microns, Sigma) were added to the resultant suspension and vortexed for 1 minute in order to lyse the cells. The extract was removed to another tube, the glass beads were washed with 300 µl of 5% TCA, and this second extract was added to the first. The mixture of both extracts was centrifuged at 3000 rpm for 10 min and the pellet, containing the proteins, was resuspended in 200 µl of 1X "high pH" Laemmli buffer (ordinary Laemmli sample buffer + 150 µl 1M Tris base / 1ml buffer). Samples were boiled for 4 min and centrifuged again at 3000 rpm for 10 min. Protein samples were transferred to a new tube, discarding the pellets. 5 to 10 µl of the samples were run in 10% SDS-PAGE gels. Proteins were transferred to nitrocellulose filters which were blocked and processed as described (9). Anti-GAL4-AD antibody (Sigma-Aldrich) for detection of the fusion proteins and secondary antibody (peroxidase-conjugated anti-rabbit IgG from ICN) were used at a 1:10,000 dilution. Detection was performed with Supersignal kit (Pierce) and bands analyzed on a Chemi-Doc apparatus (Bio-Rad). To detect TrwC expression in human cells, 293T cells were grown and transfected as explained for immunofluorescences, centrifuged, washed with PBS, and pellets kept at -80ºC. About 10^6 cells were lysed with 30 µl NP40 lysis buffer (150 mM NaCl, 50 mM Tris (pH 8), 20 mM NaF, 1% NP40, 1 mM Na_3VO_4, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 10 µg/ml leupeptin). 10 µl of supernatant were kept for protein quantitaion with Bradford. The rest was mixed with an equal volume of 2x Laemli buffer. 10-20 µl samples were boiled, run on SDS-PAGE, transferred and detected as above, except for the primary antibody which was anti-TrwC (15) used at a 1:10,000 dilution.

Database search. We searched for sequences homologous to the R388 *nic* sequence in the human genome using the "Somewhat similar sequences (blastn)" tool against the "Human genomic + transcript" database in the NCBI Blast web page (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The search was performed using the 16 nucleotide sequence 14+2, corresponding to 14 nucleotides 5' to the *nic* site + 2 nucleotides 3' to the *nic* site (see Fig. 2a).

Results

TrwC mediates site-specific recombination between bacterial and human DNA sequences.

The minimal region described as a target for *in vivo* TrwC-mediated recombination is 14+3, at *oriT1* (7). The shortest oligonucleotide that includes determinants for TrwC binding and nicking is 14+2. This segment is also enough to confer basic *oriT* function (17, 33) (Fig. 1b). We have searched for putative TrwC target sequences in the human genome (Fig. 2a). No perfect 14+2 matches were obtained. The search returned matches corresponding to shorter sequences 12+2 (four times) and 13+1 (once). A short "core" sequence, corresponding to the sequence 10+2 was found > 50 times. We repeated the search allowing for 1 mismatch within the 14+2 sequence. Seven hits were returned (Fig. 2a). All matches corresponded with either non annotated or intronic regions of the human genome. The top five human matches shown in Fig. 2a have a mismatch out of the 6+2 region, critical for TrwC nicking activity.

It is predicted that TrwC binding will be a prerequisite for targeted integration. We have also shown that TrwC-mediated site-specific recombination reaction requires the nicking and strand-transfer abilities of TrwC, which can be observed *in vitro* on oligonucleotides containing its target sequence (32). In order to determine if the sequences found in the human genome could be targets for incoming TrwC-DNA, we performed TrwC binding and strand-transfer reactions with labelled oligonucleotides including some of the sequences shown in Fig. 2a. We selected two sequences, Hu.X and Hu.5, corresponding to 15+3 sequences carrying a single mismatch in the TrwC binding site. Both putative human targets in chromosomes X and 5 were bound by TrwC roughly with the same affinity as the R388 sequence (Fig. 2b), and both acted efficiently as acceptors of the cut R388 strand donated by TrwC (Fig. 2c).

We tested several of the human sequences as TrwC targets in *in vivo* site-specific recombination assays. We designed primers amplifying ca. 400 bp fragments from the human genome, and containing the putative target sequences. We cloned these sequences at the *oriT1* locus in the recombination substrate, which carried a wild-type R388 *oriT* copy at the *oriT2* locus. Recombination was assayed as described (7). In order to confirm the requirement of an intact *nic* site for recombination, we tested a substrate carrying a full-length *oriT1* but carrying a mutation within the nic site, TCT/A to GAG/A. No recombination was observed (**Fig. 3**, compare top and bottom left panels). Constructs containing the human sequences at *oriT1* were compared to that containing a minimal *oriT1* with the canonical 14+3 R388 sequence. The results are shown in Fig. 3. TrwC is capable of catalyzing recombination between a copy of its cognate *oriT* and 15+3 sequences containing a single mismatch in the TrwC binding site (top panels 3 and 4), with roughly the same proficiency as with the canonical 14+3 sequence (top panel 2). None of the constructs containing shorter consensus sequences (12+3, 13+1 or 10+2) behaved as substrates for TrwC-mediated recombination (Fig. 3, bottom panels). Thus, there are a few sequences in the human genome which are targets for TrwC-mediated site-specific recombination, while sequences deviating from the consensus for more than 1 bp or not preserving the *nic* site are not substrates for recombination.

Localization of TrwC in human cells

In order to act as a site-specific integrase in higher organisms, TrwC must reach the nucleus by active transport. Thus, we addressed a study of TrwC localization in eukaryotic cells. Although there is no widely accepted consensus for NLS, two basic clusters of residues form the most accepted bipartite NLS: K R $X_{(10-12)}$ K K/R X K/R (11). The second cluster on its own can function as a monopartite NLS. TrwC contains two putative NLSs in its sequence: residues 53-56 (KRFR) and 171-190 (KR-X_{14}-KRTR), both within the relaxase domain.

We cloned DNA fragments coding for the relaxase (N293), the recombinase (N600) and the helicase (C774) domains of TrwC, all known to produce stable proteins products (7, 32), and full-length TrwC, into vector pCEFL under the control of the eukaryotic constitutive promoter EF1α. We transfected human 293T cells with these plasmids and confirmed by western blot that the proteins were being stably produced in the cell (data not shown). No apparent citotoxic effect was observed in the cells after 24h of *trwC* overexpression. We performed immunofluorescence microscopy to show the cellular distribution of each TrwC domain (**Fig. 4**). The relaxase and recombinase domains of TrwC (N293 and N600) located preferentially to the nucleus, while the C-terminal helicase domain was cytoplasmic, suggesting that the putative NLS were functional. However, the localization of full-length TrwC was clearly cytoplasmic.

Nuclear import/export assays in yeast

In order to corroborate the above results and to try to understand why full-length TrwC does not localize to the nucleus while shorter N-terminal fragments do, we have used a yeast-based genetic assay to look for nuclear import/export signals (36). In the import assay, test proteins are fused to LexA-Gal4AD; if the test protein enables nuclear import, LexA-Gal4AD turns on reporter genes in the yeast strain L40, which are detected by expression of beta-galactosidase and/or growth in DO-his plates. We cloned DNA fragments coding for the different TrwC domains into the pNIA vector. To verify that the fusion protein LexA-Gal4AD-TrwC was functional, we confirmed that plasmid pCMS9 (pNIA::*trwC*) efficiently complemented plasmid pSU1445 (R388 TrwC$^-$) with a similar transfer frequency as a plasmid coding for wild type TrwC (**Table 3a**). We transformed the constructs into L40 yeast cells and performed western blots using anti-Gal4AD antibody; the fusion proteins were detected in comparable amounts (data not shown). Controls for the import assay were proteins VirD2, which enters yeast and animal nuclei, and VirE2, which enters only the plant cell nucleus (18, 23). Results (**Fig. 5**) indicate

that the relaxase and recombinase domains of TrwC enter the nucleus, since they drive the fused LexA-Gal4AD to their nuclear targets, leading to expression of HIS and growth on DO-his plates, while growth was not observed with either the fused helicase domain or full length TrwC, correlating with the results obtained in human cultured cells.

The cytoplasmic localizaton of TrwC could be due to the lack of a functional NLS, but this is surprising considering that the N-terminal derivatives locate to the nucleus. Another option would be the presence of a sequence acting as a nuclear export signal (NES) within the helicase domain of the protein, which would redirect the protein back into the cytoplasm. An analysis of the TrwC sequence using NetNES (CBS, Technical University of Denmark), which predicts leucine-rich nuclear export signals (NES) in eukaryotic proteins (25) (http://www.cbs.dtu.dk/services/NetNES/), rendered no putative NES candidates; however, there is no general consensus for NES, since export pathways other than the CMR1-mediated are known to exist (20). We performed the related yeast nuclear export assay (36), implemented to detect the reverse protein transport into the cytoplasm. To this end, the pNEA vector, containing a NLS fused to LexA-Gal4AD, was used. By default, there will be nuclear localization of the transcriptional activators and HIS expression. Fusion of a NES expressing protein would result in the transport of the protein back into the cell cytoplasm and lack of expression of the reporter gene. We cloned both full-length TrwC and the helicase domain C774 in this plasmid. We checked that pCMS15 (pNEA:*trwC*) fully complemented the R388 TrwC deficient plasmid in conjugation (Table 3). The results (Fig. 5b, right panels) show that fusion to the whole length TrwC protein resulted in growth, meaning that the NLS from the vector targeted the fusion protein to the nucleus, where it remained; while fusion to the helicase domain impedes growth in DO-his plates, meaning this domain is driving the fusion protein out of the nucleus. Thus, it appears that the localisation of TrwC could be at

equilibrium between the nucleus (driven by the NLS in the relaxase domain) and the cytoplasm (driven by a NES present in the helicase domain).

Intragenic complementation of TrwC

N600, the recombinase domain of TrwC, enters the nucleus of yeast and human cells and remains stably within it. From the point of view of its possible application as an integrase for mammalian genomic engineering, it would be interesting to see whether N600 can be delivered to the recipient cell *in vivo*. If N600 can be delivered to recipient bacteria by conjugation, it can be assumed that it will be also delivered to recipient human cells by conjugation as described (Waters, 2001).

We know that C-terminal deletions of TrwC are not functional in conjugation. However, it was published elsewhere (32) that, when co-expressed in the donor bacteria, a relaxase fragment (N348) and the helicase domain C774 of TrwC could complement each other functionally to substitute TrwC in conjugation, with a frequency 10^4 lower than the wild type protein. This N348 fragment is, however, rather unstable (7). We assayed the N600 fragment, together with other TrwC fragments known to be stable, N293 and N450 (7), for their capacity to complement an R388 TrwC deficient plasmid in conjugation in the presence of the helicase domain. Results in Table 3b indicate that larger fragments N450 and N600 can functionally complement the helicase with the same efficiency as the relaxase fragment N293. It is possible that the N-terminal domain contains part of the secretion signal that would allow the protein to be delivered through the T4SS into the recipient cell, but, as shown for most T4SS, the main determinant for substrate recognition could reside in the C-terminus (4). Hence, N600 can be transferred to a recipient cell by conjugation if the helicase domain is provided in the donor cell, albeit with very low efficiency.

TrwC mutants which enter the nucleus

Since the recombinase domain of TrwC targets efficiently the human nucleus but can be transferred with very low efficiency by conjugation, it would be desirable to obtain a full-length TrwC protein which locates to the nucleus. The results from the yeast nuclear import/export assays suggested that a NES in the C-terminal domain of TrwC was responsible for the cytoplasmic localization of the protein. We reasoned that we could target TrwC to the nucleus in two ways: by adding extra NLS or by getting rid of the NES.

We constructed pNIA:TrwC derivatives carrying either an extra NLS at the C-terminus, or several NLS at its N-terminus by addition of oligonucleotides coding for the peptide IPKKKRKV in phase with *trwC* (see section "Plasmid constructions" in M&M, and Table 2). Cloning of the insert gave rise to constructs containing one, two, or five NLS, in frame with the N-terminus of TrwC. By western blot, we checked that NLS-TrwC levels were similar to wild-type, except for the construct with five NLS, which was not detected (not shown) and thus we did not use it further. All plasmids complemented R388 (TrwC-) with high efficiency (Table 3a). These TrwC derivatives were also tested for their ability to catalyze site-specific recombination. As can be observed in **Fig. 6a**, the LexA-Gal4AD-TrwC fusion protein is recombination proficient (top panel 1), and the addition of a NLS to the C-terminus of TrwC does not affect its recombination capacity (compare top panels 1 and 3; 100% colonies with blue sectors); however, addition of NLS to the N-terminus of TrwC affects its recombinase activity (Fig. 6a, bottom panels), from a mild effect when a single NLS is added (more than 70% colonies showing blue sectors, albeit smaller than in wild-type), to a stronger effect with two N-terminal additional NLS (about 40% colonies showing small blue sectors). When these plasmids were introduced into L40 yeast cells to test their cellular localization, we found that none of them showed nuclear localization, according to the lack of growth in DO-his plates (Fig. 6b, and data not shown).

Our second strategy was to obtain a TrwC mutant in the NES sequence, which would show nuclear localization, while probably maintaining its function in

conjugation and recombination. We performed random mutagenesis on plasmid pCMS9 (pNIA:*trwC*), selecting for mutants which grew in DO-his plates, as explained in M&M. In total, out of about 70,000 colonies screened, we obtained six His+ colonies. 3 out of the six plasmids had no *trwC* insert, so they represented recircularized pNIA3b molecules (which code for a LexA-Gal4AD fusion that enters the nucleus by passive diffusion; (36)). The other three constructs were introduced in *E. coli* DH5α harbouring pSU1445, a TrwC-deficient R388 derivative, to test TrwC function. Two of them were transfer-negative, and were no longer characterized. The remaining construct, named pLA44, complemented pSU1445 almost to the same level as pCMS9 (Table 3a). The His+ phenotype of pLA44 was confirmed and compared to that of TrwC; it can be observed that the mutant protein allows growth on DO-his plates as the positive control VirD2 (Fig. 6b). We also checked its recombination activity: it can be observed (Fig. 6a, top panel 2) that the mutant TrwC protein is as capable as wild-type TrwC in catalyzing *oriT-oriT* recombination.

The *trwC* insert was sequenced to determine the TrwC mutations responsible for the His+ phenotype: it carried a T to G and two G to A mutations at positions 1986, 2529 and 2710 of the *trwC* ORF, giving rise to two silent mutations, and missense mutation Ala904-Thr in TrwC respectively; in addition, presumably due to a defective oligonucleotide molecule, a CG dinucleotide was missing at position 2893 of *trwC*, producing a sense mutation which added to the *trwC* ORF a tail from the vector sequence giving rise to the extra peptide KVNSCSHGSSRSTRD after residue 964 of TrwC. In order to determine which of the two mutations was responsible for nuclear localization, a plasmid was constructed carrying only the missense mutation in TrwC (pLA66, Table 2); this protein did not enter the nucleus according to the lack of growth in DO-his plates (data not shown).

In conclusion, we have obtained a TrwC mutant which is fully proficient in conjugation and recombination and targets the nucleus, which will be presumably a useful biotechnological tool for genomic engineering of human cells.

Discussion

We previously reported that protein TrwC of the conjugative plasmid R388 is capable of promoting site-specific recombination between short *oriT* sequences (6, 7), and also to catalyse site-specific integration upon conjugative transfer into a resident *oriT* copy (10). In this work we describe the ability of TrwC to mediate recombination on specific putative human target sequences, while discriminating amongst more frequent ubiquitous sequences. We also report the competence of TrwC recombinase domain to enter the human nucleus, and the possibility to transport this domain to the recipient cell when complemented intragenically with the helicase domain of the protein in conjugation assays. Finally, we succeed in obtaining a full-length TrwC mutant which targets the nucleus while it maintains its function in conjugation and recombination. Overall, these results emphasize the features of TrwC as a site-specific integrase that could be of potential biotechnological use for the genetic manipulation of human cells.

TrwC is structurally related to AAV-Rep protein (17, 21), which catalyzes the integration of the single-stranded viral genome into a unique human sequence homologous to the viral origin of replication, containing the 16-bp Rep binding motif and the 6-bp nicking site (26). In a similar way, TrwC could catalyze the integration of incoming ssDNA into target sites present in the genome of the recipient cell; such putative targets would be short DNA sequences resembling the R388 *nic* site. We searched the human genome database and we found no matches containing the precise R388 14+2 sequence. Sequences 12+3 or 13+1 were not targets for TrwC mediated recombination with a wild type R388 *oriT* copy (Fig. 3, bottom panels), underscoring the sequence specificity of TrwC. A substrate containing either sequences 15+3 (-7) on chromosome X, or 15+3 (-10) on chromosome 5, with the mismatch lying outside of the essential nicking region, yielded similar recombination efficiency as R388 14+3 sequence (Fig. 3, top panels). TrwC-mediated recombination is affected by vector DNA replication and by local DNA

topology (6, 7), so DNA packaging in the human genome may well affect TrwC-mediated integration of foreign DNA; it has been shown that the ability of Tn7 to transpose to a putative human target site is decreased in *in vitro* assembled nucleosomes (24). Integration assays in human cells will be required to determine if any of the above mentioned human sequences works as a target for TrwC-mediated integration of foreign DNA, and if this integration has any deleterious effect on human cell physiology; although the putative targets do not affect any known coding sequence, the Hu5 target lies in an intronic region, which could affect correct splicing of the *MCC* gene.

In order to integrate exogenous DNA into these specific sites of the human genome, the protein must reach the nucleus. Although the nuclear membrane is not a permanent barrier, it has been reported that addition of a NLS to an integrase increases its integration frequency in eukaryotes (8). TrwC or its functional domains were expressed in human cell lines and their localization confirmed by immunoflluorescence (Fig. 4): the relaxase and recombinase domains of TrwC showed nuclear localization, supporting the existence of a functional NLS in the N-terminal domain of TrwC. Similar results were obtained in a yeast-based assay (Fig. 5), where TrwC fragments were expressed fused to transcriptional regulators, thus discarding nuclear entrance by passive diffusion. However, the whole length protein showed cytoplasmic localization in both assays. The results of the yeast nuclear export assay suggested the presence of a NES-like sequence in the helicase domain of the protein (Fig. 5), which could transport TrwC back into the cytoplasm. We obtained by random mutagenesis a TrwC mutant which targets the nucleus while retaining full activity in conjugation and recombination, of potential interest for future experimentation on TrwC activity as a site-specific integrase in mammals. The mutant carried a missense A904T mutation close to the C-terminus, and a sense mutation which added a peptide tail to the protein KVNSCSHGSSRSTRD. Since a plasmid carrying only the A904T mutation did not enter the nucleus, the

added tail is responsible for nuclear localization of TrwC, presumably by affecting a NES not belonging to any defined consensus.

Addition of NLS sequences to either the N- or the C-terminus of TrwC did not target the protein to the nucleus. Curiously, the progressive addition of NLS to the N-terminus of the protein did not alter its function in conjugation, but affected its recombinase activity (Table 3a and Fig. 6). This is the first time that recombinase activity can be separated from TrwC function in conjugation. This different effect could be due to a higher demand of TrwC for recombination than conjugation; in conjugation, we have observed repeatedly that very small amounts of TrwC are enough to efficiently mobilize DNA. Fusion proteins could alter TrwC function and yet this would not affect conjugation due to the high number of TrwC molecules in the cell, while recombination would be affected. A similar effect has been recently reported for conjugative coupling protein TrwB (9): to observe the effect of certain TrwB mutants in conjugation, a TrwB-limiting mating assay had to be used; otherwise, mild phenotypes are masked by the high number of TrwB molecules present in the cell.

Recombinases of bacterial or viral origin have been widely used for mammalian genomic modification (42). Among them, the only integrases known to work on naturally existing human targets are the TrwC-related AAV-Rep protein and integrases of the phiC31 family; these proteins pose several problems for genome modification, such as their low sequence specificity, viral limited DNA packaging capacity, or toxicity of the expression of the integrase in human cells (35). These problems could be overcome by the system we propose, based on *in vivo* TrwC-DNA transfer to mammalian cells by bacterial conjugation (45). The obtained TrwC nuclear mutant could mediate site-specific integration of the incoming foreign DNA into specific human targets. Conjugation is a processive mechanism which imposes no limit on the DNA length that could be transferred into human cells. *trwC* overexpression does not seem to affect cell viability or morphology; but even more importantly, since TrwC is delivered to the recipient cell attached to the DNA, there

is no need for *trwC* expression in the recipient cell, thus avoiding toxicity problems. And finally, conjugative relaxases show high sequence specificity. In the future, a bank of mutant relaxases could be obtained which target different sequences; in fact, the first such TrwC mutants have already been obtained (13). We are also addressing a mutagenesis analysis of TrwC-related relaxases to obtain recombinase-proficient mutants which could be similarly used on other possible targets present in human and mammalian genomes.

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Figure legends

Figure 1. Functional dissection of TrwC and its target *oriT* sequence. a) Map of the TrwC protein showing its functional domains. Tra, transfer (functionality in conjugation). Rel, in vitro relaxase activity. Rec, in vivo site-specific recombinase activity. Hel, DNA helicase activity. Dim, dimerization ability. Data taken from (32) and (7). b) DNA sequence of the central R388 *oriT* region (coordinates 201 to 173 from (27)). The inverted repeat IR_2 is indicated with arrows. Horizontal bars show the minimal sequence requirements for different TrwC activities: *bind*, in vitro TrwC binding on oligonucleotides; *nic*, in vitro TrwC nicking and strand-transfer activity on oligonucleotides; *recombination*, in vivo TrwC-mediated site-specific recombination; *transfer*, conjugal mobilization (data taken from (33) and (7)).

Figure 2. Putative TrwC targets in the human genome. a) DNA sequences resembling the R388 *nic* site. The minimal region required for TrwC activity is highlighted in bold. The *nic* site is indicated by a slash. Arrows show the inverted repeat recognised by TrwC. Nucleotides identical to the R388 sequence are shown in capital letters. The minimal target corresponds to the 173-190 *oriT* sequence present in plasmid pCIG1073. The mutation altering the R388 *nic* site in pCIG1110 is shown below the wild-type R388 sequence. Below, the seven human sequences including the R388 14+2 sequence with a single mismach are shown, together with shorter sequences mentioned in the text. Sequences are named indicating the human (Hu.) chromosome number and the homologous nucleotides 5'+3' with respect to the *nic* site; mismatch position is indicated in brackets. b) TrwC binding assays on (25+8) oligonucleotides containing the sequences shown in a). The amount of TrwC-N293 protein in each assay is indicated at the bottom (in ng). Arrows point to free (grey arrow) and retarded, TrwC-bound oligonucleotide (black arrow). c) TrwC strand-transfer reactions on oligonucleotides including the indicated R388 and human sequences. Arrows point to the cut product (grey arrow) or strand

transfer product (black arrow), which is quantitated at the bottom of the gel (%ST). Assays in b) and c) performed as explained in Materials and Methods.

Figure 3. Typical colonies obtained with recombination substrates containing the indicated sequences (as shown in Fig. 2a) cloned at *oriT1*. *oriT2* remains invariable, and contains the full length original R388 *oriT*. Recombination assays were done in DH5α strain in the presence of a plasmid coding for both TrwA and TrwC.

Figure 4. Localization of TrwC and derivatives in human cells. Immunofluorescence images of 293T cells transduced with plasmids coding for TrwC or the indicated TrwC segments**.** Images are shown at 40x.

Figure 5. Yeast nuclear import/export assays. **a)** Scheme of the yeast nuclear import assay. The test protein (green circle) is fused to transcriptional regulators which turn on reporter genes if driven into the nucleus, thus allowing expression of *HIS3* and *lacZ*. Selection is illustrated in the right panels: *HIS3* expression allows growth in histidine-deficient medium (-His) and *lacZ* is monitored by β-galactosidase activity in cells transferred to nitrocellulose filters with X-gal (NC+X-gal). **b)** Results of the nuclear import (left two panels) and export (right two panels) assay with TrwC and derivatives. Numbers on plates in both a) and b) are as follows: 1, pNIA:VirD2; 2, pNIA:E2; 3, pNIA:N293; 4, pNIA:C774; 5, pNIA:TrwC; 6, pNIA:N600; 7, pNEA:VirE2; 8, pNEA:TrwC; 9, pNEA:C774.

Figure 6. TrwC mutants for nuclear targeting. a) Recombination assays. DH5α cells containing the recombination substrate pCIG1030 (including *trwA*) and the plasmid coding for the indicated TrwC fusion derivative were plated on X-gal containing selective media. TrwC* refers to the TrwC mutant coded by plasmid pLA44. b) Yeast nuclear import assays (as in Fig. 5). C+ and C- refer to pNIA:VIrD2

and pNIA:VirE2, respectively. Numbers on plates indicate: 1, pNIA:TrwC; 2, pNIA:TrwC* (TrwC mutant coded by pLA44); 3, pNIA:TrwC-NLS; 4, pNIA:NLS(x2)-TrwC.

Table 1. Published plasmids used in this work.

Plasmid	Description	Reference
pCEFL	eukaryotic expression vector	(43)
pCIG1028	*oriT1-oriT2* recombination substrate	(7)
pCIG1030	As pCIG1028 + *trwA*	(7)
pCIG1051	pET3a::*trwC(N450)*	(7)
pCIG1073	As pCIG1028 with *oriT1*(173-190)	(7)
pCIG1099	pET3a::*trwC(N600)*	(7)
pCMS13	pKK223-3::*oriT(63-330)mut*[1]	(7)
pET:trwA	pET3a::*trwA*	(7)
pET3:trwAC	pET3a:P_{trwA}-*trwA-trwC*	(10)
pET29:trwAC	pET29c:P_{trwA}-*trwA-trwC*	(10)
pNEA3b	*lexA-SV40NLS-gal4AD* fusion	(36)
pNEA:VirE2	control - for nuclear export	(36)
pNIA3b	*lexA-gal4AD* fusion	(36)
pNIA:VirD2	control + for nuclear import	(36)
pNIA:VirE2	control - for nuclear import	(36)
pSU19	cloning vector	(1)
pSU1445	R388:Tn*5*tac1 in *trwC*	(28)
pSU1483	pKK223-3::*trwC*	(15)
pSU1534	pHG327::*trwC(C774)*	(32)
pSU1600	pET3a::*trwC(N293)*	(7)
pSU1621	pET3a::*trwC*	(17)

[1] Mutation affecting the *nic* site (TCT/A to GAG/A change)

Table 2. Bacterial plasmids constructed in this work.

		Construction [1]		
Plasmid	**Description**	**Vector**	**Insert**	**Digestion/oligonucleotides (5´-3´)**
Recombination substrates				
pCIG1110	*oriT1*(63-330*mut* [2])	pCIG1028	pCMS13	AACTCTAGAACCCAATGCGCATAGCG AACAAGCTTCCTCTCCCGTAGTGTTAC
pCIG1116	*oriT1* HuX 15+3*(-7)* [3]	pCIG1028	human DNA	CCATCTAGATTAGACACAGGCTCTACTCACACAG TACAAGCTTAAAAATTCAACACAGCCTCTAAGTG
pCIG1117	*oriT1* Hu5 15+3*(-10)* [3]	pCIG1028	human DNA	CCATCTAGATCTAAGATGCAGTAAGATCCCAGAC TACAAGCTTTCAAGATTAGTGAGCAAGAAATGTG
pCIG1122	*oriT1* Hu18 (10+2) [3]	pCIG1028	human DNA	CCATCTAGACCTTGAACCTATTCTGCCCATA TACAAGCTTTCAAGGCTCTTGATGTTTGAGA
pCIG1126	*oriT1* Hu5b (13+1) [3]	pCIG1028	human DNA	CCATCTAGAAGCTATGCACAACAGCATGG TACAAGCTTAATCCCAATATTTGACCACCA
pCIG1127	*oriT1* Hu7 (12+3) [3]	pCIG1028	human DNA	CCATCTAGACCTGGCGATAGAGCAAGACT TACAAGCTTCTGACCACCTGCTCCAAAAT
Plasmids for intragenic complementation assays				
pCIG1070	pSU19::TrwC (N450)	pSU19	pCIG1051	XbaI / BamHI
pCIG1086	pSU19::TrwC	pSU19	pSU1621	XbaI / BamHI
pCIG1103	pSU19::TrwC (N600)	pSU19	pCIG1099	XbaI / BamHI
pLA35	pSU19::TrwC (N293)	pSU19	pSU1600	XbaI / BamHI
Plasmids for yeast nuclear import/export assays				
pCIG1133	pNIA::TrwC (N600)	pNIA3b	pSU1621	ACCGGATCCCGATGCTCAGTCACATGGT CCAGAATTCACTCGATGGCCTTGGTTTG
pCMS2	pNIA::TrwC (N293)	pNIA3b	pSU1621	ACCGGATCCCGATGCTCAGTCACATGGT ACCGAATTCAGCTGAAATCTATGCCGAG

		Construction [1]		
Plasmid	**Description**	**Vector**	**Insert**	**Digestion/oligonucleotides (5´-3´)**
pCMS5	pNIA::TrwC-NLS	pNIA3b	pSU1621	ACCGGATCCCGATGCTCAGTCACATGGT CCAGAATTCTACCTACCTTTCTTTCCGGCCTCCATGCC
pCMS9	pNIA::TrwC	pNIA3b	pSU1621	ACCGGATCCCGATGCTCAGTCACATGGT ACCGAATTCACCTTCCGGCCTCCATGCC
pCMS10	pNIA::TrwC (C774)	pNIA3b	pSU1621	CCAGGATCCTTGGAGCCGTCTATAAC ACCGAATTCACCTTCCGGCCTCCATGCC
pCMS15	pNEA::TrwC	pNEA3b	pCMS9	ACCGGATCCCGATGCTCAGTCACATGGT CCACTCGAGACCTTCCGGCCTCCATGCC
pCMS16	pNEA::TrwC (C774)	pNEA3b	pCMS10	CCAGGATCCTTGGAGCCGTCTATAAC CCACTCGAGACCTTCCGGCCTCCATGCC
pLA44	pNIA::TrwC* [4]	pCMS10	pCMS9	mutagenic PCR [5] GGCTGGCGGTTGGGGGTTA ACCGAATTCACCTTCCGGCCTCCATGCCGCG
pLA66	pNIA::TrwC (A904T)	pCMS10	pLA44	ACCGGATCCCGATGCTCAGTCACATGGT ACCGAATTCACCTTCCGGCCTCCATGCC
pMTX719	pNIA::NLS(x5)-TrwC	pCMS9	oligos [6]	GATCCCCAAGAAGAAACGGAAGGT GATCACCTTCCGTTTCTTCTTGGG
pMTX720	pNIA::NLS(x2)-TrwC	pCMS9	oligos [6]	GATCCCCAAGAAGAAACGGAAGGT GATCACCTTCCGTTTCTTCTTGGG
pMTX726	pNIA::NLS-TrwC	pMTX719	none	BamHI and religation
Plasmids used for immunofluorescences				
pLA14	pCEFL::TrwC	pCEFL	pET29:trwAC	ACCAAAGCTTATGCTCAGTCACATGGTATT ACCAGGATCCTTACCTTCCGGCCTCCA
pLA27	pCEFL::TrwC (N293)	pCEFL	pCMS3	BamHI / EcoRI
pLA28	pCEFL::TrwC (N600)	pCEFL	pCIG1133	BamHI / EcoRI
pLA29	pCEFL::TrwC (C774)	pCEFL	pCMS10	BamHI / EcoRI

[1] First column lists the vector plasmids; second column lists the plasmids from which the inserts were obtained, and third column indicates either the restriction enzymes used for cloning, or the oligonucleotides used for PCR amplification of the desired fragment, with the restriction sites underlined.

[2] Mutation affecting the *nic* site (TCT/A to GAG/A change)

[3] Human sequences in the indicated human chromosomes (Hu X, 5, 18, or 7). n + n' indicates the extend of the consensus sequence around the *nic* site; variations from consensus are indicated in brackets (see text for details and nomenclature).

[4] TrwC*: TrwC mutant obtained by random mutagenesis. It carries missense mutation A904T and the additional peptide KVNSCSHGSSRSTRD after residue 964 of TrwC.

[5] This PCR reaction was performed under mutagenic conditions, as described in Materials and Methods. The PCR fragment was digested with BamHI and EcoRI and ligated into the same sites of the pCMS10 backbone.

[6] NLS sequences were added to the N-terminus of TrwC by oligonucleotide hybridization and insertion at BamHI site of pCMS9.

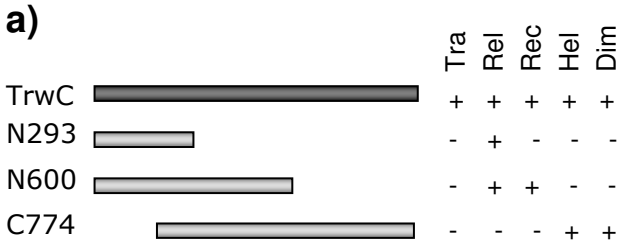
Table 3. Transfer frequencies of R388 *trwC* mutant pSU1445 when complemented by different TrwC derivatives [1]

Plasmids	***TrwC protein***	***Transfer freq*** [2]
a) complementation by TrwC fusion proteins		
pSU1483	TrwC	$1,0 \times 10^{-1}$
pCMS9	LexA-Gal4AD-TrwC	$4,0 \times 10^{-1}$
pCMS15	LexA-NLS-Gal4AD-TrwC	$6,0 \times 10^{-1}$
pCMS5	LexA-Gal4AD-TrwC-NLS	$4,0 \times 10^{-1}$
pMTX720	LexA-Gal4AD-NLSx2-TrwC	$1,1 \times 10^{0}$
pMTX726	LexA-Gal4AD-NLS-TrwC	$1,3 \times 10^{0}$
pLA44	LexA-Gal4AD-TrwC *mut*	$1,3 \times 10^{-1}$
pNIA3b	none	$< 10^{-7}$
b) relaxase-helicase intragenic complementation		
pCIG1086+pSU1534	TrwC + C774	$2,1 \times 10^{-1}$
pLA35+pSU1534	N293 + C774	$1,1 \times 10^{-5}$
pCIG1070+pSU1534	N450 + C774	$1,1 \times 10^{-5}$
pCIG1103+pSU1534	N600 + C774	$3,1 \times 10^{-5}$
pSU1534	C774	$< 10^{-7}$

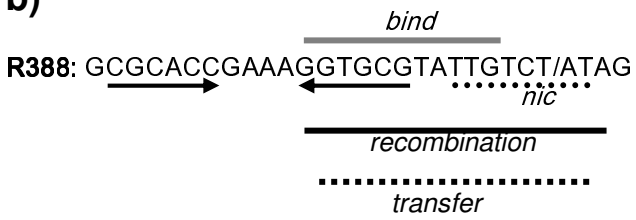
[1] Matings were performed as explained in M&M. Donor strains carried plasmid pSU1445 (R388 TrwC-) and the plasmid(s) indicated in the first column, which code for the TrwC derivatives indicated in the second column.

[2] Transfer frequencies, expressed as trasnconjugants per donor cell. Results represent the mean of 3-9 independent experiments.

a)


Tra
Rel
Rec
Hel
Dim
TrwC
+ + + + +
N293
- + - - -
N600
- + + - -
C774
- - - + +

b)


bind
R388: GCGCACCGAAAGGTGCGTATTGTCT/ATAG
nic
recombination
transfer

a)

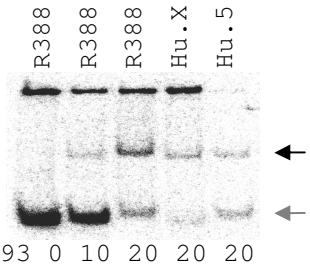
bind

Minimal target: **GGTGCGTATTGTCT/AT**A

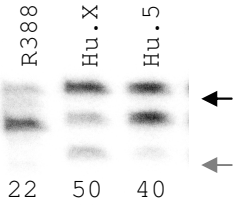
nic

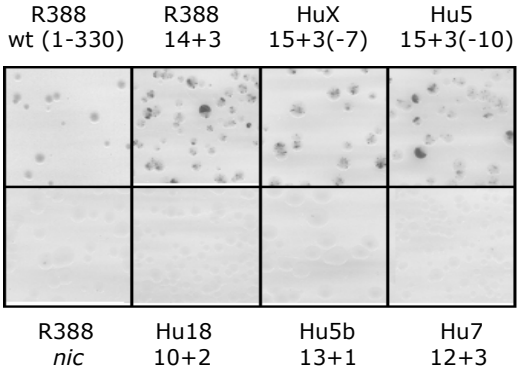
R388: GCGCACCGAAA**GGTGCGTATTGTCT/AT**AGCCCA
***nic*:** GCGCACCGAAA**GGTGCGTATTG**gag**/AT**AGCCCA
Hu.X: GgaattttcgA**GGTGCGT**t**TTGTCT/AT**AaatgA 15+3(−7)
Hu.5: agtattaGAtA**GGTG**t**GTATTGTCT/AT**AtatgA 15+3(−10)
Hu.13:aatatagcAtA**GGTGC**a**TATTGTCT/AT**Actaat 15+3(−9)
Hu.15:GCatctCtgcA**GGTGC**t**TATTGTCT/AT**ttgtat 15+2(−9)
Hu.11:ttaggggttgg**GGTG**g**GTATTGTCT/AT**cttttA 14+2(−10)
Hu.3: aaGCggaGcAt**GGTGCGTATT**t**TCT/AT**gGttag 14+2(−4)
Hu.10:ctatACCcccA**GGTGCGTATTGTCT/**t**T**ccCagg 14+2(+1)
Hu.5b:taGtctgttgta**GTGCGTATTGTCT/A**ctatatg 13+1
Hu.7: ttaCACatttAtt**TGCGTATTGTCT/AT**AtCCac 12+3
Hu.18: GtGtctgcAggaaat**CGTATTGTCT/AT**caaagt 10+2

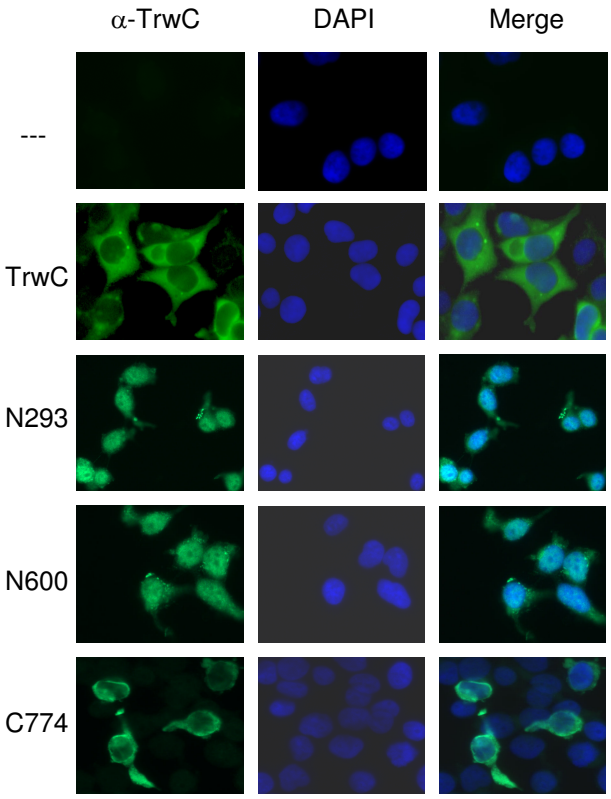
b)



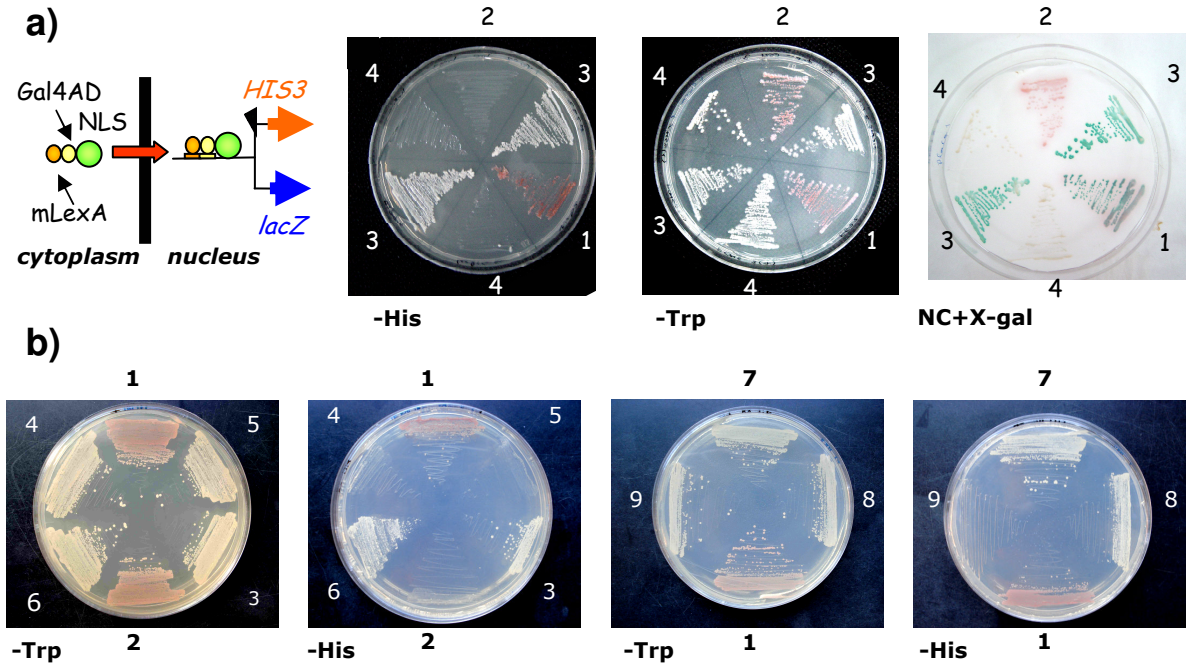
c)

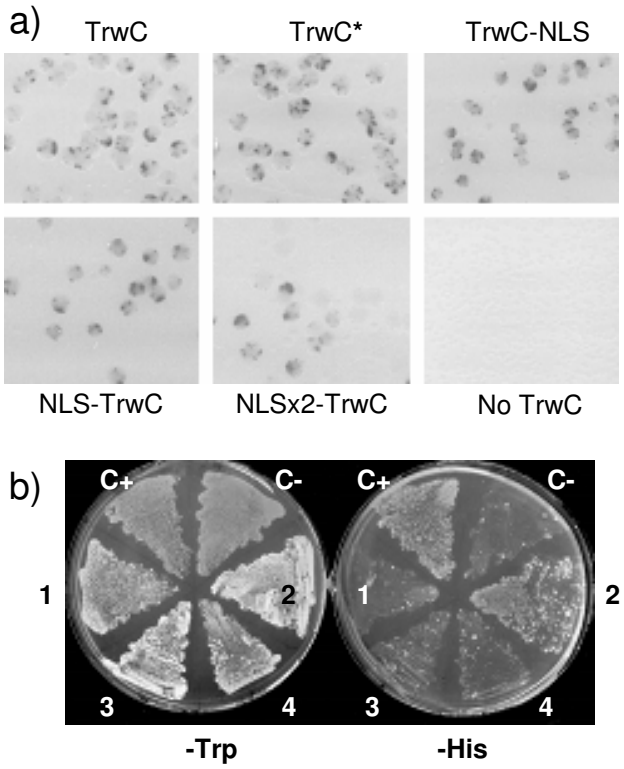



R388
wt (1-330)
R388
14+3
HuX
15+3(-7)
Hu5
15+3(-10)
R388
nic
Hu18
10+2
Hu5b
13+1
Hu7
12+3


α-TrwC
DAPI
Merge

TrwC
N293
N600
C774


a)
Gal4AD
NLS
mLexA
HIS3
lacZ
cytoplasm
nucleus
2
4
3
3
1
4
-His
2
4
3
3
1
4
-Trp
2
4
3
3
1
4
NC+X-gal
b)
1
4
5
6
3
2
-Trp
1
4
5
6
3
2
-His
7
9
8
1
-Trp
7
9
8
1
-His


a)
TrwC
TrwC*
TrwC-NLS
NLS-TrwC
NLSx2-TrwC
No TrwC
b)
C+
C-
C+
C-
1
2
1
2
3
4
3
4
-Trp
-His