Demonstration that the Group II Intron from the Clostridial Conjugative Transposon Tn5397 Undergoes Splicing In Vivo

ADAM P. ROBERTS,1 VEIT BRAUN,2 CHRISTOPH VON EICHEL-STREIBER,2 AND PETER MULLANY1*

Department of Microbiology, Eastman Dental Hospital for Oral Health Care Sciences, University College London, University of London, London WC1X 8LD, United Kingdom, 1 and Verfügungsgebäude für Forschung und Entwicklung, Institut für Medizinische Mikrobiologie und Hygiene, Johannes-Gutenberg-Universität, 55101 Mainz, Germany2

Received 21 August 2000/Accepted 21 November 2000

Conjugative transposons are genetic elements that encode their own integration, excision, and transfer functions. They are remarkably promiscuous and are capable of being transferred across large phylogenetic distances. They are important clinically, as they are one of the major vectors involved in the spread of antibiotic resistance among bacterial pathogens. There are several recent reviews describing the properties of conjugative transposons (2, 17, 19, 20). Tn5397 is a conjugative transposon isolated from the gram-positive anaerobic pathogen Clostridium difficile strain 630 (15, 16). Tn5397 encodes tetracycline resistance via the tet(M) gene and has been shown to be transferable by a conjugation-like process from C. difficile 630 to Bacillus subtilis CU2189 and back to C. difficile CD37 (16). It has also been shown to be able to transfer between C. difficile strains (16). Furthermore, Tn5397 has been shown to readily transfer from a B. subtilis donor to a Streptococcus acidominimus recipient in a model oral biofilm community, indicating that the element is likely to be able to transfer to a new host in the natural environment (18). Physical and genetic analysis has shown that Tn5397 is related to the extensively studied conjugative transposon Tn916 (7, 15, 16, 24). There are, however, some important differences. The ends of the two elements are completely different, with the xis and int genes of Tn916 being replaced by a gene called tndX in Tn5397. TndX is a member of the family of large resolvase/invertase proteins. This protein is responsible for the insertion and excision of Tn5397 (24).

The other major difference between Tn916 and Tn5397 is that Tn5397 contains a group II intron, inserted into a gene that is almost identical to orf14 from Tn916 (15). The Tn5397 version of this gene is termed orf14+. Group II introns are a class of genetic elements that were first discovered in the genomes of eukaryotic organelles in fungi and in plants. They are categorized by their secondary structure, which is essential for splicing (12). As well as being capable of splicing, group II introns can also transpose to allelic sites (a process called homing) at high frequency and to ectopic sites at a much lower frequency (12). Some group II introns encode a multifunctional protein, with maturase, reverse transcriptase, and endonuclease activities (4). This protein acts on both RNA and DNA and, together with the catalytic RNA of the intron, forms a ribonucleoprotein (RNP) particle which is required to promote homing, splicing, and transposition activities (3, 4, 27, 28).

Over the past few years, group II introns have been found in a number of different bacteria (5, 6, 8, 10, 13, 15, 26). In most cases, however, splicing and mobility have not been reported to occur in vivo. The main exception to this has been the demonstration that the Lactococcus lactis intron LlitrB, inserted into the putative relaxase gene (litrB) of the conjugative element pRS01, is spliced and is capable of transposition (13, 14). Furthermore, splicing of this intron was found to be required for conjugal transfer of pRS01 (13). As the group II intron within Tn5397 is inserted into a gene shown to be required for conjugative transfer of the related conjugative transposon Tn916 (21), the work described in this paper was designed to see if the intron is spliced in vivo and if splicing is required for conjugal transfer of Tn5397. We demonstrate that the intron is spliced but that splicing is not a requirement for conjugal transfer of the host conjugative transposon.

MATERIALS AND METHODS

Bacterial strains and plasmids. All bacterial strains and plasmids used are listed in Table 1. C. difficile and B. subtilis were routinely grown on or in brain heart infusion (BHI) agar or broth (Oxoid, Basingstoke, U.K.). C. difficile strains for RNA preparations were grown in Wilkins-Chalgren medium (Oxoid, Basingstoke). E. coli strains were grown on or in Luria-Bertani (LB) agar or broth. C. difficile strains were grown anaerobically in an anaerobic chamber (Don Whitley Scientific) (10% hydrogen, 10% carbon dioxide, and 80% nitrogen), and B. subtilis and E. coli were grown aerobically. Antibiotics were used at concentrations of 10 μg/ml for tetracycline, 25 μg/ml for kanamycin, 25 μg/ml for...
Spliced mRNA can be detected from \textit{C. difficile} containing Tn5397. The Tn5397 group II intron is contained within \textit{orf14*}, which is almost identical to \textit{orf14} from Tn916 (15, 24). The genetic organization of this region is shown in Fig. 1. In order to determine if the intron is spliced, the primers I5 and I6 were used in an RT-PCR on RNA isolated from \textit{C. difficile} 630. A product of 330 bp was obtained from cDNA generated with random primers (Fig. 2A) and with cDNA template generated with a specific primer from the coding strand. However, no PCR product was observed when cDNA generated with a specific primer from the noncoding strand was used as the template, indicating, as expected, that splicing only occurs in RNA identical to the coding strand (results not shown). The 330-bp PCR product was sequenced, and it consisted of an in-frame ligation of the two flanking exons, the splice site being located exactly as predicted from the secondary structure of the intron (15). That the intronic open reading frame (ORF) is transcribed was confirmed by RT-PCR using primers I1 and I2 (results not shown). The same results were obtained when the cells were harvested at early, mid-, or late exponential phase (results not shown).

Nonspliced intron-exon borders can be detected from \textit{C. difficile} containing Tn5397. To determine if unspliced intron was also present, RT-PCR with primer pairs I5 and I3 was used to amplify the 5' intron-exon junction, and primers I4 and I6 were used to amplify the 3' intron-exon junction. The results are shown in Fig. 2A and show that both the 5' and 3' intron-exon junctions are present in the \textit{C. difficile} RNA. These results were confirmed by DNA sequence analysis.

Spliced and nonspliced RNA can also be detected in \textit{B. subtilis}. RNA was prepared from the \textit{B. subtilis} strain BS6A (18), which contains Tn5397, to see if the intron is spliced in this host. Essentially the same experiments as those described for \textit{C. difficile} 630 were carried out, and the RT-PCR gave products of identical size as those shown in Fig. 2A for the \textit{intron} in \textit{C. difficile} 630. Again, DNA sequencing of the PCR products confirmed that they represented ligated exons and intron-exon borders, indicating that both spliced and nonspliced RNA is present in \textit{B. subtilis} (results not shown).

Determination of relative amounts of spliced and nonspliced intron. A dilution series of cDNA template prepared from \textit{C. difficile} strains 630 and FM1A (Table 1) were used in RT-PCRs to determine the relative amounts of 5' intron-exon junction, 3' intron-exon junction, and ligated exons. The re-
results of this analysis are shown in Table 2. The results show that the 5′ intron-exon junction was apparently present at about a 10^6-fold-lower amount than the 3′ intron-exon junction. There also seems to be as much 3′ intron-exon junction as spliced intron. This could mean that the splicing of the 5′ intron occurs 10^6 times faster than that of the 3′ intron. Alternatively, some of the intron intermediates may get degraded before splicing is complete. Lariat is more stable than linear RNA, so if splicing were aborted before finishing, the 3′ half (with the lariat) would be expected to persist longer than the 5′ half.

**Deletion of part of the intronic ORF prevents splicing but does not prevent conjugal transposition of Tn5397.** An intron mutant was constructed in *B. subtilis* strain BS6A (see Materials and Methods). The mutant was designated BS16A. When RT-PCR using primers I5 and I6 was performed, a PCR product of 478 bp was detected (lane 1, Fig. 2B). This product did not correspond to the size expected of spliced intron. DNA sequencing of the product showed that it resulted from mispriming with primer I5. Therefore, we could not detect splicing in the mutant intron.

Previous work in our laboratory has shown that Tn5397 produces a circular form and that this molecule is likely to be the conjugative transposition intermediate (24). In order to determine if the *B. subtilis* strain containing the intron mutant was still capable of excision and producing the circular form of the transposon, primers reading out from the ends of the element were used for PCR, as in Wang et al. (24). Using these primers, a PCR product would only be produced if the element circularizes and the left and right ends are ligated together. A PCR product of the expected size (272 bp) corresponding to the ligated ends of the transposon was produced in *B. subtilis* BS6A (*B. subtilis* strain containing wild-type Tn5397) and BS16A (BS6A::Tn5397 IntronΔkan) (Fig. 2C). DNA sequencing of the PCR product showed that the two ends of Tn5397 had been ligated together with a GA dinucleotide at the joint between the ends of the element, as is the case in the wild-type Tn5397 (24). These results show that splicing is not required for production of the circular form of the element.

The *B. subtilis* strains BS6A and BS16A were used as donors in a filter mating experiment with *C. difficile* CD37 as the recipient. Transconjugants arose at a frequency of 2 × 10^-7 per donor, a frequency similar to that previously reported for the transfer of Tn5397 from *B. subtilis* to *C. difficile* (16, 24). All transconjugants still contained the IntronΔkan allele (confirmed by PCR and DNA sequencing; not shown). Therefore, preventing splicing does not prevent conjugal transfer.

**DISCUSSION**

In this work, we have demonstrated that the intron from Tn5397 splices in vivo in both *C. difficile* and *B. subtilis*. This is an important observation as, although group II introns have recently been found in a number of different bacteria (5, 6, 8, 10, 13, 15, 26), only the *Lactococcus lactis* group II intron L1.ItrB has previously been showed to be capable of in vivo splicing (13). The same group II intron has also been found independently in the putative relaxase gene of a conjugative element inserted in the chromosome of *L. lactis* 712 (22). Furthermore, the L1.ItrB intron is more closely related to the mitochondrial group II introns than to the other introns found in bacteria (13). The Tn5397 intron falls within a group composed of only bacterial introns. A secondary-structure comparison of the introns also showed that the lactococcal intron belongs to subgroup IIA and the Tn5397 intron to subgroup IIB (S. Zimmerly, personal communication). Therefore, this is

| C. difficile strain | Primers used | Lowest dilution giving positive result |
|---------------------|--------------|---------------------------------------|
| 630                 | I5 + I3     | 10^-2                                 |
|                     | I5 + I6     | 10^-2 - 10^-10                        |
|                     | I4 + I6     | 10^-8 - 10^-9                         |
|                     | I5 + I3     | 10^-2                                 |
|                     | I5 + I6     | 10^-7 - 10^-10                        |
|                     | I4 + I6     | 10^-8 - 10^-10                        |
| FM1A                | I5 + I3     | 10^-2                                 |
|                     | I5 + I6     | 10^-7 - 10^-10                        |
|                     | I4 + I6     | 10^-8 - 10^-10                        |

*Only the 330-bp product corresponding to ligated exons was observed using primer pair I5 and I6.*
the first demonstration of in vivo splicing in a member of this family of group II introns. Most of the group II introns found in bacteria (including the mitochondrion-like L1ItrB intron) have been found associated with genes that are proven or likely to be involved in DNA mobility or contained within mobile genetic elements (5, 6, 8, 10, 13, 15, 26). Splicing of the lactococcal intron L1ItrB was required for conjugal transfer of the host element (13). In contrast, however, our results show that splicing of the intron is not required for conjugative transfer of Tn916, even though the gene interrupted by the intron in Tn9397 has been shown to be required for conjugal transfer of the related conjuga
tive transposon Tn926 (2). As the regions concerned with conjuga
tion in Tn916 and Tn9397 are very closely related, we expected that the orf14* gene product would also be required for transfer of Tn9397. However, as the intron is located near the 3′ end of the gene, lack of splicing would only result in the translated protein’s losing the last 23 amino acids, which may not be required for full function. Furthermore, we cannot rule out the possibility that splicing is required in C. difficile but not in B. subtilis due to different host factors which may substitute for the function provided by Orf14*.

As well as being located within genes, at least one group II intron has been shown to be inserted in an intergenic region within the class II transposon TnMer1 (8). Therefore, there appears to be no actual requirement for all group II introns to be spliced. However, retention of this ability increases the number of sites into which group II introns can transpose without having an adverse effect on the viability of the host cell. Therefore, it is not unexpected that these introns have retained their splicing ability.

Demonstration that the Tn9397 intron splices in both B. subtilis and C. difficile, and the results of Matsuura et al. (11) showing that the L. lactis intron is capable of in vivo splicing in both L. lactis and E. coli, indicates that group II introns are func
tional in a wide range of distantly related hosts. Zimmerly and coworkers (personal communication) have shown that there has been extensive horizontal gene transfer of group II introns in the past. The frequent association of these introns with broad-host-range conjugal elements, such as Tn9397 and pRS01, provides a means by which some of these introns are dispersed to distantly related hosts, and the minimal requirement for specific host factors allows the introns to be retained in their new hosts.

In conclusion, we have shown that the group II intron within Tn9397 is capable of splicing in both B. subtilis and C. difficile but that splicing is not required for conjugal transfer of Tn9397.

ACKNOWLEDGMENTS

A.P.R. was the recipient of a BBBCRC studentship. The work undertaken in one of the laboratories (P.M.) was funded by the Wellcome Trust. The collaboration between the two groups (P.M. and C.v.E.S.) was generously funded by the British Council.

We thank Steven Zimmerly for helpful discussions.

REFERENCES

1. Anagnostopoulous, G., and J. Spizizen. 1961. Requirements for transforma
tion in Bacillus subtilis. J. Bacteriol. 81:741–746.
2. Clewell, D., S. A. Flanagan, and D. D. Jaworski. 1995. Unconstrained bacterial promiscuity: the Tn916/Tn1545 family of conjugal transposons. Trends Microbiol. 229:229–236.
3. Cousineau, B., S. Lawrence, D. Smith, and M. Belfort. 2000. Retrotranspo-
sition of a bacterial group II intron. Nature 404:1081–1021.
4. Cousineau, B., D. Smith, S. Lawrence-Cavanagh, J. E. Mueller, J. Yang, D. Mills, D. Manias, G. Dunny, A. M. Lambowitz, and M. Belfort. 1998. Ret-
rotransposition of a bacterial group II intron: mobility via complete reverse splic
ing, independent of homologous DNA recombination. Cell 94:451–462.
5. Ferat, J.-L., M. Le Gour, and F. Michel. 1994. Multiple group II self-
splicing introns in mobile DNA from Escherichia coli. C.R. Acad. Sci. Paris Ser. 3:317:141–148.
6. Ferat, J.-L., and F. Michel. 1993. Group II self-splicing introns in bacteria. Nature 363:358–361.
7. Hachler, H., F. H. Kayser, and B. Berger-Bachi. 1987. Homology of a transposable tetracycline resistance determinant of Clostridium difficile with Streptococcus (Enterococcus) faecalis transposon Tn916. Antimicrob. Agents Chemother. 31:1033–1038.
8. Huang, C.-C., M. Narita, T. Yamagata, Y. Itoh, and G. Endo. 1999. Structure analysis of a class II transposon encoding mercury resistance of the Gram-
positive bacterium Bacillus megaruberus MB1, a strain isolated from Mi
namata Bay, Japan. Gene 234:361–369.
9. Hundsberger, T., V. Braun, M. Weidmann, P. Leukel, M. Sauerborn, and C. von Eichel-Streiber. 1997. Transcriptional analysis of the genes ted/B-E of the pathogenicity locus of Clostridium difficile. Eur. J. Biochem. 244:735–742.
10. Knoop, V., and A. Brennicke. 1994. Evidence for a group II intron in Escher
icchia coli inserted into a highly conserved reading frame associated with mobile DNA sequences. Nucleic Acids Res. 22:1167–1171.
11. Matsuura, M., R. Saldanha, M. Hongwen, H. Wang, J. Yang, G. Mohr, S. Cavanaugh, G. M. Dunny, M. Belfort, and A. M. Lambowitz. 1997. A bacterial group II intron encoding reverse transcriptase, maturation, and DNA endo
nuclease activities: biochemical demonstration of maturation activity and in
erision of new genetic information within the intron. Genes Dev. 11:2910–2955.
12. Michel, F., and J.-L. Ferat. 1995. Structure and activities of group II introns. Annu. Rev. Biochem. 64:335–341.
13. Mills, D. A., L. L. Mckay, and G. Dunny. 1996. Splicing of a group II intron in the conjugal transfer of pRS01 in lactococci. J. Bacteriol. 178:3531–3538.
14. Mills, D. A., D. Manias, L. L. Mckay, and G. Dunny. 1997. Homing of group II intron from Lactococcus lactis subsp. lactis ML3. J. Bacteriol. 179:6107–6111.
15. Mullany, P., M. Pallen, M. Wilks, and S. Tabaqchali. 1996. A group II intron in a conjugal transposon from the Gram-positive bacterium, Clostridium difficile. Gene 174:145–150.
16. Mullany, P., M. Wilks, I. Lamb, C. Clayton, B. Wren, and S. Tabaqchali. 1990. Genetic analysis of a tetracycline resistance determinant from Clostridium difficile and its conjugal transfer to and from Bacillus subtilis. J. Gen. Microbiol. 136:1343–1349.
17. Rice, L. B. 1998. Tn916 family of conjugal transposons and dissemination of antimicrobial resistance determinants. Antimicrob. Agents Chemother. 42:1871–1877.
18. Roberts, A. P., J. Pratten, M. Wilson, and P. Mullany. 1999. Transfer of a conjugal transposon, Tn9397, in a model oral biofilm. FEMS Microbiol. Lett. 177:63–66.
19. Selwyn, A. N., N. B. Shoemaker, A. M. Stevens, and L. L. Hing-Yew. 1995. Conjugal transposons: an unusual and diverse set of integrated gene transfer elements. Microbiol. Rev. 59:579–590.
20. Scott, J. R., and G. G. Churchward. 1995. Conjugal transposition. Annu. Rev. Microbiol. 49:367–397.
21. Senghas, E., J. M. Jones, M. Yamamoto, C. Gawron-Burke, and D. B. Clewell. 1988. Genetic organization of the bacterial conjugal transposon Tn916. J. Bacteriol. 170:245–249.
22. Shearman, C. H., J. J. Gordon, and G. Gasson. 1996. Splicing of a group II intron in a functional transfer gene of Lactococcus lactis. Mol. Microbiol. 21:45–53.
23. Tieu-Cust, P., G. Gerhard, T. Lambert, and P. Courvalin. 1985. In vivo transfer of genetic information between Gram positive and Gram negative bacteria. EMBO J. 4:3583–3587.
24. Wang, H., A. P. Roberts, D. Lyras, J. I. Rood, M. Wilks, and P. Mullany. 2000. Characterization of the ends and target sites of the novel Tn916-like conjugal transposon Tn9397 from Clostridium difficile: demonstration that excision and circularization are mediated by TnDX, a member of the large resolvase family, J. Bacteriol. 182:3775–3783.
25. Yanisch-Perron, G., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13, mpl8, and pUC19 vectors. Gene 33:103–119.
26. Yeo, C. C., J. M. Tham, M. W. C. Yap, and C. L. Poh. 1997. Group II intron from Pseudomonas alcaligenes NCIB 9807 (P25X): entrapment in plasmid RPl and sequence analysis. Microbiology 143:2833–2840.
27. Zimmerly, S., H. Guo, P. S. Perlman, and A. M. Lambowitz. 1995. Group II intron mobility occurs by target DNA-primed reverse transcription. Cell 82:543–554.
28. Zimmerly, S., H. Guo, R. Eskes, J. Yang, P. S. Perlman, and A. M. Lambowit
z. 1995. A group II intron is a catalytic component of a DNA endonu
clase involved in intron mobility. Cell 83:529–538.