Nucleotide sequence and proposed secondary structure of *Columnnea* latent viroid: a natural mosaic of viroid sequences

R.Hammond^{1*}, D.R.Smith¹, and T.O.Diener^{1,2}

¹Microbiology and Plant Pathology Laboratory, USDA-ARS Beltsville, MD 20705 and ²Center for Agricultural Biotechnology and Department of Botany, University of Maryland, College Park, MD 20742, USA

Received June 22, 1989; Revised and Accepted October 4, 1989

EMBL accession no. X15663

ABSTRACT

The *Columnnea* latent viroid (CLV) occurs latently in certain *Columnnea erythrophae* plants grown commercially. In potato and tomato, CLV causes potato spindle tuber viroid (PSTV) - like symptoms. Its nucleotide sequence and proposed secondary structure reveal that CLV consists of a single-stranded circular RNA of 370 nucleotides which can assume a rod-like structure with extensive base-pairing characteristic of all known viroids. The electrophoretic mobility of circular CLV under nondenaturing conditions suggests a potential tertiary structure. CLV contains extensive sequence homologies to the PSTV group of viroids but contains a central conserved region identical to that of hop stunt viroid (HSV). CLV also shares some biological properties with each of the two types of viroids. Most probably, CLV is the result of intracellular RNA recombination between an HSV-type and one or more PSTV-type viroids replicating in the same plant.

INTRODUCTION

Columnnea latent viroid (CLV) was discovered by transfer of nucleic acid preparations from symptomless *Columnnea erythrophae* leaves obtained from a commercial nursery in Beltsville, Maryland to Rutgers tomato plants. The viroid causes symptoms in Rutgers tomato similar to, but less severe than, those produced by the severe strain of potato spindle tuber viroid (PSTV) (1). In potato (*Solanum tuberosum*, cv. Katahdin), CLV causes symptoms typical of those incited by PSTV (T. O. Diener, D. R. Smith, and R. A. Owens, unpublished observations). *C. erythrophae* is an epiphyte from Central America; no viroids, however, could be isolated from about 120 samples of *Columnnea* spp. collected in Costa Rica (T.O. Diener and R. Gamez, unpublished observations). Sampling of selected *Columnnea* cultivars obtained from European nurseries, however, indicated the presence of a CLV-related viroid (R. W. Hammond and D. R. Smith, unpublished observations). The origin of CLV is unknown.

The faster migration of this viroid on nondenaturing polyacrylamide gels in comparison to PSTV and its limited sequence homology to PSTV — 20-40% by

nucleic acid hybridization -- suggested that it was a new viroid (1). In this paper, we describe the molecular cloning of the cDNA of CLV and present its complete nucleotide sequence and most probable secondary structure.

MATERIALS AND METHODS

Purification of CLV

CLV was maintained in Columnea erythrophae Decne ex. Hoellet and propagated in tomato (Lycopersicon esculentum Mill cv. Rutgers). Low molecular weight RNA (2M LiCl-soluble fraction) was extracted from tomato or Columnea leaves 3 to 6 weeks after inoculation and used for further inoculation or for purification of CLV by gel electrophoresis (2). Nucleic acids were analyzed by nondenaturing and denaturing gel electrophoresis. For electrophoresis under nondenaturing conditions, 20 ug of tomato low molecular weight RNAs were fractionated in a 20% polyacrylamide gel for 48 hours at 4 C in TAE (0.04 M Tris-acetate, 0.002M EDTA, pH 7.2) (3). For analysis under denaturing conditions, 0.5 - 1.0 ug of purified viroid RNAs were fractionated in a 5% polyacrylamide gel containing TBE (0.089 M Tris-borate, 0.089 M boric acid, 2.5 mM EDTA, pH 8.3) and 8M urea (run for 2 hours at 50 - 55 C). Both types of gel were stained with ethidium bromide and photographed.

Host range studies

Small Gynura aurantiaca (Bl.) DC plants (5 - 6 - leaf stage), cucumber (Cucumis sativus L., cv. Suvo) and tomato (Lycopersicon esculentum Mill cv. Rutgers) plants in the cotyledonary stage, and tobacco (Nicotiana tabacum L., cv. Xanthi and Xanthi nc.) plants (3 - leaf stage) were inoculated with low molecular weight RNA preparations from CLV - infected Columnea plants as described (4). Inoculated plants were kept at 30 - 35 C and observed for symptom development. Three to 6 weeks p.i., low molecular weight RNA preparations from inoculated plants were analyzed for the presence of CLV by dot-blot assay (5), using a CLV-specific RNA probe transcribed from pCol.3.12. (diagrammed in Figure 2).

Synthesis and cloning of CLV cDNA

Double-stranded cDNA was synthesized from CLV RNA extracted from Columnea erythrophae. The nucleotide sequence of certain regions of the viroid had been determined by enzymatic sequencing of the purified CLV RNA (M. C. Kiefer, unpublished results). Oligonucleotide primers complementary to the RNA at positions 152-165 (primer 2, 5' ATTACTCTGTCTG 3'), and at positions 34-51 (col P1, 5' GCATGGCTGCAGGGTCAG 3') were used for the synthesis of single-stranded cDNA in separate reactions (6). Second-strand cDNA was

synthesized by standard methods using the Klenow fragment of DNA polymerase I (6). Aliquots of the cDNA were digested with PstI and SmaI restriction enzymes and fragments were ligated into PstI/SmaI-digested pUC9 (7). Ligation reaction mixtures were then used to transform Escherichia coli strain JM83 to ampicillin resistance.

The presence of identical cDNA restriction endonuclease termini in the two sets of cDNA clones allowed us to construct a full-length cDNA of CLV. The 370 bp insert was cloned into the PstI site of pUC9, resulting in clone p.Col.3.12 (illustrated in Figure 2). Alternatively, the 370 bp insert was cloned into the PstI site of pSP64 and the resulting recombinant was used to generate high specific activity RNA probes.

Plasmid analysis

For small scale plasmid DNA preparations, the method of Birnboim and Doly (8), and for large scale plasmid preparations, the method supplied by Promega Biotec, Inc. (Madison, WI 53711)* were used. Restriction enzymes were obtained from Bethesda Research Laboratories (Gaithersburg, MD 20877).

Nucleic acid sequencing

Typically, 10 ug of plasmid DNA were digested with an appropriate restriction endonuclease and the 5'-termini were then labelled with [32 P] dATP (New England Nuclear, Wilmington, DE 19898; 800 Ci/mmol) (9). Labelled DNA fragments were separated in acrylamide gels, excised, and eluted. DNA was sequenced by the procedure of Maxam and Gilbert (10) with a modification (11). The most stable secondary structure of the RNA was determined by the computer program of Zuker and Stiegler (12).

Infectivity studies

Prior to inoculation, double-stranded plasmid DNA of the construction pCol.3.12 (illustrated in Figure 2) was digested with PstI to release the viroid insert, and 10 ug/plant (at a concentration of 1 ug/ul) of the digested plasmid was inoculated onto the cotyledons of 6-day old Rutgers tomato plants. Three weeks following inoculation, infectivity was determined by nucleic acid dot hybridization (5), using a high specific activity SP6 generated RNA probe prepared by transcription of the recombinant pSP64 plasmid (13).

* Mention of trademark, proprietary product or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of the products or vendors that may be suitable.

RESULTS

Electrophoretic mobility of CLV.

Low molecular weight RNAs isolated from tomato plants infected with PSTV, chrysanthemum stunt viroid (CSV), tomato planta macho viroid (TPMV), tomato apical stunt viroid (TASV), or CLV were analyzed by nondenaturing polyacrylamide gel electrophoresis (Figure 1A). CLV (lanes 3 and 5) migrates faster than either CSV (lane 4, 354 nt.), PSTV (lane 2, 359 nt.), TPMV (lane 6, 360 nt.), or TASV (lane 7, 360 nt.). In contrast, the electrophoretic mobility of CLV under denaturing conditions (Figure 1B, lane 2) in relation to PSTV (Figure 1B, lanes 1 and 3) is slower. Typically, the migration of circular viroid molecules under nondenaturing conditions is dependent not

Figure 1. Electrophoretic mobility of CLV and selected other viroids during nondenaturing (A) and denaturing (B) polyacrylamide gel electrophoresis, as described in Materials and Methods. A) lane 1, uninfected control; lane 2, PSTV; lanes 3 and 5, CLV; lane 4, CSV; lane 6, TPMV; lane 7, TASV. B) lanes 1 and 3, PSTV; lane 2, CLV.

only on the size of the molecule but also on its compact secondary structure, while the comparative migration of circular viroid molecules under fully denaturing conditions may be used to estimate the relative size of the molecule. While the slower migration of CLV on a denaturing gel indicates that it is larger than PSTV, its change in relative position on a nondenaturing gel suggests a somewhat atypical secondary or tertiary structure.

Molecular cloning of CLV cDNA

Difficulties encountered in direct RNA sequencing of CLV by standard enzymatic techniques and oligonucleotide-primed dideoxy sequencing were suggestive of extensive secondary structure (M. C. Kiefer, unpublished results). However, these preliminary experiments yielded sequence information which was used to design oligonucleotide primers for cDNA synthesis.

Initially, primer 2 (described in MATERIALS AND METHODS, homologous to positions 152-165 of the RNA) was used to prime first-strand cDNA synthesis. Double-stranded cDNA was prepared and digested with S1 nuclease and subsequently blunt-end ligated into the *Sma*I site of pUC9. One of the longest cDNA clones obtained, pCol.P2.1, contained a 330 base pair cDNA copy of CLV,

Figure 2. Restriction maps of cloned cDNA copies of CLV and the strategy for DNA sequencing. Unit length CLV is shown as a thick line in pCol.3.12. Hatched bars show the cloned cDNAs representing partial copies of the genome. Relevant restriction enzyme sites are indicated. Numbers correspond to the nucleotide positions on circular CLV (shown in Figure 3). The direction and extent of sequence determination are indicated by arrows. E=*Eco*RI; H=*Hind*III; Ha=*Hae*III; P=*Pst*I; Pv=*Pvu*II; S=*Sma*I; X=*Xho*I. * vector restriction sites not present in the CLV sequence.

from residues 200 - 370 and 1-160 (Figure 2). Restriction endonuclease mapping and DNA sequence analysis revealed that the CLV sequence contained convenient PstI and SmaI restriction sites, useful for cloning into pUC-type plasmid vectors. cDNA synthesis was then performed using primer 2 and primer col P1 to prime first strand cDNA synthesis in separate reactions. The double-stranded cDNA resulting from each of these syntheses was digested with PstI and SmaI and ligated into similarly digested pUC9. Representative cDNA clones obtained from col P1 and primer 2 reactions are shown in Figure 2 as pCol.1 and pCol. 2, respectively. A full-length copy of CLV was constructed by ligation of PstI/SmaI fragments from pCol.1 and pCol.2 into the PstI site of pUC9; it is represented by pCol.3.12. (Figure 2).

Infectivity of CLV cDNAs

After constructing a recombinant plasmid containing a full-length copy of CLV (see Figure 2, pCol.3.12), the infectivity of this cloned sequence was determined. Double-stranded plasmid DNA was digested to completion with PstI in order to release the viroid fragment. The total digestion mixture (10 ug DNA/plant) was inoculated onto tomato cotyledons and infectivity was assayed by the appearance of characteristic disease symptoms (stunting and epinasty). The presence of viroid-homologous sequences in inoculated plants was verified by nucleic acid spot hybridization of leaf sap prepared from the bioassay plants by using a high specific activity radioactive CLV-specific RNA probe. The cloned sequence illustrated in Figure 2 (pCol.3.12) was highly infectious in tomato (data not shown) and therefore represents a complete copy of CLV. Sequence analysis of the viroid progeny isolated from the bioassay plants established their sequence identity to the DNA inoculum used (data not shown).

Nucleotide sequence and secondary structure of CLV

The complete nucleotide sequence of CLV was determined from sequence analysis of its cDNA (pCol.3.12), and from its sequence the most likely secondary structure was derived (Figure 3). The circular RNA consists of 370 nucleotide residues (71 A, 105 G, 110 C, 84 U) which can potentially form a typical viroid-like secondary structure consisting of 121 base pairs (73 G:C, 36 A:U, 12 G:U). Sequence analysis of several cDNA clones revealed heterogeneity from

Figure 3. Nucleotide sequence and presumed secondary structure of CLV. Sequence heterogeneity among cDNAs is indicated by boxed nucleotides at positions 28, 291, and 363. The numbering of residues follows the convention established for PSTV. The cloned sequence (pCol.3.12) used for infectivity studies is represented by the major sequence shown to illustrate the secondary structure.

pCol.3.12 at three residues, positions 28, 291, and 363, as indicated in Figure 3. CLV, like all known viroids, contains an inverted repeat on either side of the upper strand center (nts. no. 86 - 94 and 109 - 117) which can anneal in analogy with PSTV into a hairpin (hairpin I) during thermal denaturations (14). Like all known viroids, except hop latent viroid (15), CLV contains a polypurine stretch (nts. 52 - 65).

Comparison with other viroids

Comparison of the nucleotide sequence of CLV with those of other viroids reveals that CLV consists of extensive stretches of nucleotide sequences present in other viroids, which are interrupted by sequences that are unique to CLV. Sequence similarities between CLV and some members of the PSTV - group of viroids, PSTV (16), TPMV (17), and TASV (17), and the type member of the hop stunt viroid group of viroids, hop stunt viroid (HSV; 18) are illustrated in Figure 4. Both the upper and lower portions of the central conserved region (CCR) are identical with those of HSV (Figure 5A); the left and right terminal domains (for definition of viroid domains, see ref. 19) are homologous with those of PSTV and TASV, respectively (Figure 5B); and the lower portion of the pathogenicity domain closely resembles that of TPMV. Sequences unique to CLV are present in the variable domain and in a 13-nucleotide insert 3' adjacent to the upper portion of the pathogenicity domain (* in Figure 4; Figure 5C). The pathogenicity and variable domains are to the left and right of the CCR (encompassing both the upper and lower strands), respectively. In common with all known viroids, the upper central conserved region of longer-than-unit length CLV strands can form a palindrome.

Host range studies

Because the primary structure of CLV contains sequence motifs characteristic

Figure 4. Schematic diagram of CLV illustrating the sequence homologies to other viroids. ■, PSTV; □, TPMV; ▨, TASV; ▩, HSV; ▧, polypurine stretch; → ←, inverted repeats; *, unique insert within PSTV sequence homology. Lft T = left terminal domain; P = pathogenicity domain; CCR = central conserved region; V = variable region; Rt T = right terminal domain.

A. Central conserved region (CCR)

Upper CCR

CLV: 94 GGAGCCCCGGGGCAACU 110
 HSV: 73 GGAGCCCCGGGGCAACU 89

Lower CCR

CLV: 261 GACGGACCGGUGGCAUCACC 281
 HSV: 210 GACGGACCGGUGGCAUCACC 230

B. Terminal regions

Left terminal region

CLV: 331 GGGCGAGGGUGUUAGCCUUGGAACCGCAGUAGGUUCCUCGGAACUAAACUCUGGUUCCUGUGGUACACACCGUACC 39
 PSTV: 320 GGGCGAGGGUGUUAGCCUUGGAACCGCAGUAGGUUCCUCGGAACUAAACUCUGGUUCCUGUGGUACACACCGUACC 39

Right terminal region

CLV: 153 AGACAGGAGUAAUCCCGAGCUGAAACAGGGUUUUCACCCUCCUUCUUCUGGUUCCUCCUCU 216
 PSTV: 149 AGACAGGAGUAAUCCCGAGCUGAAACAGGGUUUUCACCCUCCUUCUUCUGGUUCCUCCUCU 212

C. Unique insert

CLV: 72 GGAG-AGCCG/AAGAGCGGUCUCA/GGA 96
 PSTV: 72 GGAGGAGCCG/----UUCAG----/GGA 89

Figure 5. Similarities of the nucleotide sequence of CLV with those of other viroids. Boxed nucleotides indicate sequence heterogeneity in CLV.

of both PSTV group and HSV group viroids, it was interesting to determine whether CLV is able to replicate and cause symptoms in host plants typically used with viroids of the PSTV group or with HSV. Table 1 shows that CLV is able to replicate and cause disease in Gynura aurantica, which is a host of PSTV, but not of HSV, and in cucumber, which is a host of HSV, but not of PSTV. In tomato, CLV resembles PSTV by replicating and causing symptoms, whereas HSV is able to replicate, but not cause symptoms in tomato.

Table 1. Host range of CLV and selected other viroids.^a

Viroid	Tomato Repl. Sympt.		Gynura Repl. Sympt.		Cucumber Repl. Sympt.		Tobacco Repl. Sympt.	
CLV	+	+	+	+	+	+	-	-
HSV _g	+	-	-	-	+	+	-	-
PSTV	+	+	+	+	-	-	+	-
TPMV	+	++	+	-	N.D.		N.D.	

^aData for HSV_g from ref. 26; other data, original determinations.

N.D.= not determined ; Repl. = replication ; Sympt. = symptoms

DISCUSSION

Examination of the nucleotide sequence and most likely secondary structure of CLV reveals typical viroid features. CLV consists of a highly base-paired, covalently closed circular, rod-like RNA of 370 nucleotides, in which short base-paired regions are interrupted by bulge and internal loops; it contains an oligopurine stretch at its usual position on the left side of the upper strand; and a central conserved region, whose upper strand portion is flanked by an inverted repeat.

In common with all known viroids, longer-than-unit length CLV strands can assume a palindromic structure composed of the upper-strand portion of the central conserved region and the adjoining inverted repeats. This palindromic structure is similar to, but of somewhat lower thermodynamic stability than, that possible with PSTV. The latter structure has been proposed to represent a putative cleavage-ligation site involved in viroid processing (20). Recent results with oligomeric constructs of viroid cDNAs and their RNA transcripts are compatible with this model (21, 22), but also indicate the existence of one or more alternative processing sites (22, 23).

Comparison of the nucleotide sequence of CLV with those of other viroids reveals that CLV is composed of three elements (see Figure 4):

a) sequences that are identical to or closely resemble those of PSTV-type viroids. These are primarily located at the left and right terminal domains of CLV, and in its pathogenicity domain; b) sequences that are identical with those of HSV, encompassing the central conserved region; and c) sequences unique to CLV, located at various positions in the molecule but extensively in its variable domain. The chimeric structural properties of CLV are reflected in its biological properties: Like PSTV-group viroids, but unlike HSV, CLV causes disease in Rutgers tomato; like HSV, but unlike PSTV-group viroids, CLV replicates and causes disease in cucumber (Table 1). Whether the chimeric structure of CLV is a consequence of extensive in vivo RNA recombination between PSTV-group and HSV-like viroids replicating in the same host plant or whether CLV evolved independently is unknown. In the latter case, however, the striking sequence similarities between CLV and the other known viroids would represent a case of convergent structural evolution. Although convergent evolution in terms of function is common, convergence on structure is not (24). There are, in fact, no examples in which convergent evolution has led to close similarities in structure and sequence (25). Thus, the chimeric nature of CLV most likely is a consequence of extensive in

vivo RNA recombination, the possibility of which has been postulated previously (19).

Because no viroids could be detected in Columnea species collected from their natural habitat, the original host of CLV is unknown. It is conceivable, however, that chance transfer of an HSV variant present in an ornamental or crop plant [such as grapevine (26-28), citrus spp. (29-31), or cucumber (32-34)], as well as transfer of one or more PSTIV-group viroids (such as PSTIV from potato or TASV or TMV from tomato) from plants grown in a nursery into neighboring Columnea plants could provide the starting point for RNA recombination, that would eventually lead to CLV. The vegetative propagation of ornamental Columnea plants would assure that any viroid that had entered the plants at an earlier time would be maintained and amplified during propagation.

Finally, knowledge of the primary and presumed secondary structure of CLV does not explain its aberrant electrophoretic migration under nondenaturing conditions (Figure 1). Given the relatively large size of CLV, its abnormally fast migration in a nondenatured state (Figure 1A) could be a consequence of a particularly compact structure. The total number of basepairs, as well as the ratios of G:C to A:U or G:U basepairs, however, is not substantially different from those of other viroids, such as PSTIV, CSV, or citrus exocortis viroid CEV (14). Folding of the CLV molecule into a compact tertiary structure cannot be excluded on the basis of present knowledge, but is not believed to occur with other viroids (35).

Acknowledgments. The authors would like to acknowledge the technical assistance of Michelle Greyerbiehl, Marilyn Hale and Kristina Dobrowolska. We thank Susan M. Thompson for assistance with preparation of figures. We thank Drs. Candace Collmer and John Hammond for constructive comments on the manuscript.

*To whom correspondence should be addressed

References

1. Owens, R.A., Smith, D.R. and Diener, T.O. (1978) Virology 89, 388-394.
2. Schumacher, J., Sanger, H.L. and Riesner, D. (1983) EMBO J. 2, 1549-1555.
3. Loening, U. E. (1967) Biochem. J. 102, 251-257.
4. Diener, T. O., Hadidi, A., and Owens, R.A. (1977) In: Methods in Virology (K.Maramorosch and H. Koprowski, eds.) 6, 185-217.
5. Owens, R. A. and Diener, T. O. (1981) Science 213, 670-672.
6. Maniatis, T., Fritsch, E.F. and Sambrook, J.C. (1982) Molecular Cloning. Cold Spring Harbor Laboratory, New York.
7. Viera, J. and Messing, J. (1982) Gene 19, 259-268.

8. Birnboim, H.C. and Doly, J. (1979) *Nucleic Acids Res.* 7, 1513-1523.
9. Ruther, U., Koenen, M., Otto, K. and Muller-Hill, B. (1981) *Nucleic Acids Res.* 9, 4087-4098.
10. Maxam, A.M. and Gilbert, W. (1980) *Methods in Enzymol.* 65, 499-560.
11. Smith, D.R. and Cabro, J.M. (1980) *Nucleic Acids Res.* 8, 2255-2274.
12. Zuker, M. and Stiegler, P. (1981) *Nucleic Acids Res.* 9, 133-148.
13. Melton, D.A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. and Green, M. R. (1984) *Nucleic Acids Res.* 12, 7035-7056.
14. Riesner, D. and Gross, H. J. (1985) *Ann. Rev. Biochem.* 54, 531-564.
15. Puchta, H., Ramm, K., and Sanger, H.L. (1988) *Nucl. Acids Res.* 16, 4197-4216.
16. Gross, H.J., Domdey, H., Lossow, C., Jank, P., Raba, M., Alberty, H. and Sanger, H.L. (1978) *Nature (London)* 273, 203-208.
17. Kiefer, M.C., Owens, R.A. and Diener, T.O. (1983) *Proc. Natl. Acad. Sci. USA.* 80, 6234-6238.
18. Ohno, T., Takamatsu, N., Meshi, T. and Okada, Y. (1983) *Nucleic Acids Res.* 11, 6185-6197.
19. Keese, P. and Symons, R.H. (1985) *Proc. Natl. Acad. Sci. USA.* 82, 4582-4586.
20. Diener, T.O. (1986) *Proc. Natl. Acad. Sci. USA* 83, 58-62.
21. Tsagris, M., Tabler, M., Mühlbach, H.-P. and Sanger, H.L. (1987) *EMBO J.* 6, 2173-2183.
22. Hammond, R. W., Diener, T. O. and Owens, R. A. (1989) *Virology* 170, 486-495.
23. Sanger, H.L., Tabler, M., and Tsagris, M. (1988) 5th Internatl. Congress Plant Pathology, Kyoto, Japan, Abstracts, p. 40.
24. Lewin, R. (1985) *Science* 227, 1020.
25. Bannister, J.V. and Parker, M.W. (1985) *Proc. Natl. Acad. Sci. USA.* 82, 149-152.
26. Sano, T., Ohshima, K., Hataya, T., Uyeda, I., Shikata, E., Chou, T.-G., Meshi, T., and Okada, Y. (1986) *J. Gen. Virol.* 67, 1673-1678.
27. Rezaian, M.A., Koltunow, A.M. and Krake, L.R. (1988) *J. Gen. Virol.* 69, 413-422.
28. Puchta, H., Ramm, K. and Sanger, H.L. (1988) *Nucleic Acids Res.* 16, 2730.
29. Sano, T., Hataya, T., Sasaki, A. and Shikata, E. (1986) *Proc. Japan Acad.* 62B, 325-328.
30. Sano, T., Hataya, T. and Shikata, E. (1988) *Nucleic Acids Res.* 16, 347.
31. Diener, T.O., Smith, D.R., Hammond, R.H., Albanese, G., LaRosa, R., and Davino, M. (1988) *Plant Disease* 72, 691-693.
32. Van Dorst, H.J.M. and Peters, D. (1974) *Neth. J. Pl. Path.* 80, 85-96.
33. Sano, T., Uyeda, I., Shikata, E., Ohno, T. and Okada, Y. (1984) *Nucleic Acids Res.* 12, 3427-3434.
34. Puchta, H., Ramm, K. and Sanger, H.L. (1988) *Nucleic Acids Res.* 16, 8171.
35. Gross, H.J. and Riesner, D. (1980) *Angewandte Chemie, International Ed.* 19, 231-243.