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A single-strand specific endonuclease activity copurifies with overexpressed T5 D15 exonuclease

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ABSTRACT

The T5 D15 exonuclease purified from an overproducing strain of *E. coli* was shown to possess a low level of endonucleolytic activity specific for single-stranded DNA when assayed with 1–10 mM Mg^{2+} as co-factor. Endonuclease activity on double-stranded circular DNA could not be detected under these conditions. Nicked circular DNA was first gapped by the enzyme's exonucleolytic activity, creating a single-stranded region. This gapped substrate was then endonucleolytically cleaved and rapidly degraded. We show that a gapped and not a nicked substrate is required for this activity as previously suggested (Moyer, R. W. and Roth, C. T. 1977, J. Virol. 24, 177–193). The single-strand endonuclease activity could be selectively suppressed by using low concentrations of Mg^{2+} as co-factor (< 1 mM), thus allowing nicked double-stranded circular DNA to be gapped to a single-stranded circular species. We also report on sequence similarities between the T5 exonuclease and several prokaryotic DNA polymerases.

INTRODUCTION

T5 and its close relatives represent a unique group of viruses in that the double-stranded linear DNA genome is comprised of one single DNA chain 120 000 nucleotides long hybridised to a an interupted (nicked) complementary sequence. These nicks can be sealed in vitro with DNA ligase and the resultant DNA may be transfected into competent cells (1). The presence of large single-stranded regions has been detected in replicating T5 DNA (2). These regions may be important in the formation of the transcription-replication complex (3).

The D15 exonuclease is thought to be part of the bacteriophage T5 transcription-replication enzyme complex (3). It is required for transciption of late T5 genes (4) and for normal DNA replication (5). Moyer and Rothe (6) isolated the exonuclease from phage-infected cells and demonstrated an associated endonuclease activity which required DNA with a pre-existing nick as a substrate.

The T5 induced DNA polymerase (gene D9) has extensive sequence homology to *E. coli* DNA polymerase I (7) and the T5 D15 exonuclease has homology with the amino terminus of *E. coli* DNA Pol I (8). Thus, it may be inferred that together the T5 D15 and D9 gene products fulfil a similar role in phage T5 as that carried out by DNA polymerase I in uninfected *E. coli*.

The single-stranded regions produced during replication may also be involved in the regulation of late gene transcription (4). Isolated T5 exonuclease can degrade DNA at the rate of 20–30 nucleotides per second (8) and so may be involved with creating such single-stranded regions. The proposal that the D15 exonuclease can cleave across a nicked double-stranded DNA substrate seems incongruous with such a role as this would lead to fragmentation of the phage DNA (6).

We have recently reported the overexpression, isolation and some properties of the coliphage T5 gene D15 exonuclease (8). We have constructed an improved T5 D15 exonuclease overproducing strain and further investigated the properties of the enzyme. We found a low level of single-strand specific endonuclease activity repeatedly co-purifies with the exonuclease under a variety of conditions.

MATERIALS AND METHODS

Enzymes and biochemicals

Restriction enzymes were from New England Biolabs. T4 polynucleotide kinase was supplied by United States Biochemicals (Cleveland, Ohio, U.S.A.). Oligonucleotides were prepared using an Applied Biosystems 380B DNA Synthesizer. Analytical agarose gel electrophoresis was performed as in (14). The synthetic lambda promotor sequence was constructed by shotgun ligation of the following oligodeoxynucleotides; PL1 (5′-dAATTAGATAACCATCTGCGGTGATAAAT-3′), PL2 (5′-dGAGATAATTTATCACCGCAGATGGTTATCT-3′), PL3 (5′-dTATCTCTGGCGGTGTTGACATAAATACC-3′), (PL1, 3, and 5) form the upper strand), PL4 (5′-dGCCAGTGGTATTTATGTCAACACCGCCA-3′), PL5 (5′-dACTGGCGGTGATACTGAGCACATCAG-3′) PL6 (5′-dAATTCTGATGTGCTCAGTATCACC-3′).

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Cloning

Oligonucleotides PL1-PL6 were phosphorylated and cloned into the EcoRI site of pUC19 by shotgun ligation. This promotor cartridge recreates the λP_L sequence described by Fiers (9). The sequences of the oligonucleotides were chosen so as to recreate only one EcoRI site on cloning into a vector EcoRI site (see Fig 1). The recreated restriction site is positioned downstream of the promotor, such that fragments cloned into the EcoRI site would be under control of the λP_L. Recombinant plasmids were transformed into JM83(λ) cells. Plasmid minipreparations were made and the clones containing the λP_L orientated so as to initiate transcription toward the P_{lac} i.e. across the multiple cloning site, were identifed by double-digest with restriction enzymes EcoRI and PvuII. Positive clones (designated pJONEX4) produce fragments of 2364, 230 and 183 bp.

The construct M13T5SAC carrying the T5 D15 exonuclease gene has been described by us previously (8). The D15 gene was excised from this construct on a SacI/HindIII fragment. The gene was then cloned into SacI/HindIII treated pJONEX4. A recombinant carrying the T5 exonuclease gene was designated pJONEX44 (Fig 1). This plasmid was then transformed into M72(λ) cells for production of T5 exonuclease using the heat inducible λP_L, cI857 repressor system (9).

Transfections

Competent cells were prepared by the calcium chloride procedure (10) and either used directly or made 20% (v/v) in glycerine and stored frozen at −80°C. *E. coli* SMH50 were used for all M13 transfections (11). The cell lines M72(λ) and JM83(λ) were used for transformation of plasmids containing the λP_L sequence (12).

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AATTAGATAACCATCTGCGGTGATAAAT 5´      PL1  duplex a
  3 TCTATTGGTAGACGCCACTATTTAATAGAG   PL2

TATCTCTGGCGGTGTTGACATAAATACC 5´      PL3  duplex b
    3 ACCGCCACAACTGTATTTATGGTGACCG   PL4

ACTGGCGGTGATACTGAGCACATCAG 5´        PL5  duplex c
   3  CCACTATGACTCGTGTAGTCTTAA       PL6
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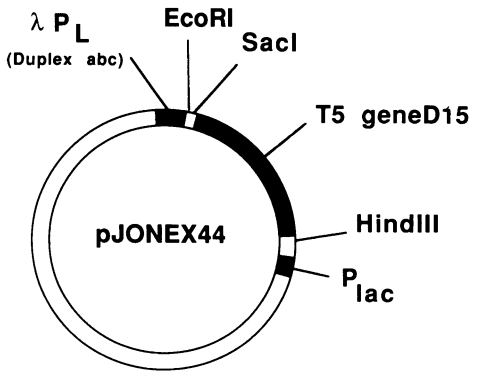

Fig. 1. Construction of plasmid pJONEX44 for the expression of T5 exonuclease. The oligonucleotide pairs (forming duplexes a, b, and c) were annealed together and ligated into the Eco RI site of pUC19. The overhanging 5′ end of duplex **a** (oligonucleotide PL1) is AATT, which ligates into the vector's Eco RI site. The next nucleotide in the sequence of PL1 is an A residue. Thus when this end of the oligonucleotide duplex is ligated into an Eco RI site the restriction site is not recreated as the new sequence is ...GAATTA... . Recombinants carrying the promotor element in the desired orientation were designated as pJONEX4. A SacI-HindIII fragment carrying the T5 exonuclease gene was ligated into pJONEX4 to yield the final construct pJONEX44 as described in Materials and Methods.

T5 exonuclease overproduction and isolation

The exonuclease was isolated from heat induced M72(λ)/pJONEX44 cells exactly as described for the isolation of the same enzyme from M72(λ)/pJON45 (8). Attempts to separate the endo- from the exonuclease activity included the following methods: FPLC on Mono Q (anion exchanger); chromatography on hydroxyapatite, on P 11 phosphocellulose at pH 9.3, on SP (sulfopropyl)-Sephadex C-25, on CM (carboxymethyl) Sephadex (cation exchangers), on Phenyl Sepharose CL-4B (hydrophobic interaction chromatography), on controlled pore glass and on Sephadex G75 under native and denaturing conditions; ultrafiltration on Amicon 30; isoelectrofocussing in the presence of 5 M urea and renaturation of SDS-PAGE separated protein.

Biological assay for single-strand specific endonuclease

The assay takes advantage of the fact that both single (SS) and double-stranded circular M13 DNA (RFIV) are transfective. Thus, a mixture of RFIV DNA of one phenotype and SS DNA of another phenotype give rise to transformants with either (but not both) phenotypes. The system we use exploits the ΔM15 *E. coli* strains/M13mp phage lacZ α-complementation system previously described (10). Phenotypes (colourless and blue) are screened by plating on X-gal/IPTG indicator plates. Thus, exposure of the mixture of RFIV and SS DNA to a single-strand specific nuclease before transfection would result in clones of only one phenotype, that of the double-stranded DNA, being obtained. For the assay double-stranded closed circular (RFIV) DNA was prepared in vitro as described (13). Single-stranded M13 DNAs were isolated as in (14). Double-stranded M13mp18 DNA (blue phenotype on indicator plates, 10 μg) was mixed with single-stranded M13mp2TAA DNA (colourless phenotype, 5 μg) in 50 μl of 50 mM Tris·HCl, pH 8, 50 mM NaCl, 8 mM $MgCl_2$. T5 exonuclease (1 μg) was added and the reaction incubated at 37°C for 30 min. A similar experiment was performed using double-stranded M13mp2TAA, colourless phenotype (14) and single-stranded M13mp18 DNA (blue

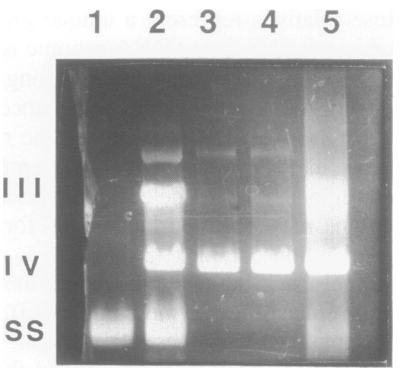

Fig. 2. The action of T5 exonuclease on a mixture of linear duplex (RFIII), closed-circular duplex (RFIV) and single-stranded circular (SS) DNA. Lane 1, circular single-stranded M13 marker. Lane 2, mixture of RFIII, RFIV and SS DNA. Lanes 3−5 show the products obtained after reaction with 600, 60 and 6 ng T5 exonuclease, respectively. Conditions as decribed in Materials and Methods. The position of RFIII, RFIV, and SS DNAs are indicated by III, IV and SS at the right of the figure.

phenotype). Blanks were performed with no added enzyme. Dilutions of 1:500 were made and used to transfect competent SMH50 cells, which were then plated on X-gal/IPTG indicator plates as described (14). The results are summarised in Table I.

Agarose gel assay for single-strand specific endonuclease

The reaction mixtures contained double-stranded linear (RFIII), closed-circular (RFIV) and single-stranded circular M13mp18 DNA (1.2 μg of each in 20 μl of 50 mM glycine KOH, pH 9.3, 10 mM $MgCl_2$ buffer). T5 exonuclease (6−600 ng) was added and the reactions were incubated for 30 min at 37°C. The reactions were terminated by addition of 10 μl 50 mM EDTA/50% glycerol. Samples of 10 μl were run on a 1% agarose gel. Results are shown in Fig 2.

Effect of Mg^{2+} concentration on T5 exonuclease gapping of nicked DNA substrates

Double-stranded nicked DNA was first desalted by ultrafiltration using an Amicon Centricon device. The assay buffer contained 50 mM glycine/KOH pH 9.3 and the DNA concentration was appoximately 80 μg/ml. Reactons were performed on 10 μl aliquots of DNA in buffer as above at final Mg^{2+} concentrations of 10, 1, 0.1, and 0.01 mM with 15 ng T5 exonuclease, incubated for 60 min at 37°C, mixed with an equal volume of dye mix and analysed by electrophoresis. These results are shown in Fig 3.

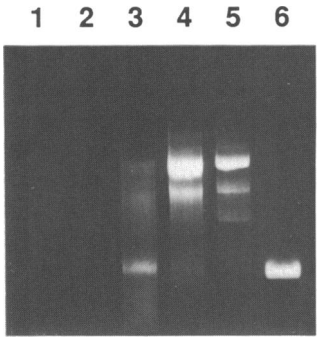

Fig. 3. The influence of Mg^{2+} concentration on the reaction of T5 exonuclease with nicked DNA. Lanes 1−4 show the products obtained at Mg^{2+} concentrations of 10, 1, 0.1 and 0.01 mM respectively. Lanes 5 and 6 show markers of double-stranded nicked and single-stranded circular DNA respectively. Conditions as described in Materials and Methods.

The time-course of the reactions with Mg^{2+} concentrations of 10 mM and 0.1 mM were investigated. The reactions (50 μl, 4 μg DNA in buffer as above) were preheated at 37°C for 5 min. Then T5 exonuclease (15 ng and 75 ng in the 10 mM and 0.1 mM Mg^{2+} reactions, respectively) was added, the reaction mixtures were vortexed, spun briefly and incubated further at 37°C. After starting the reactions aliquots were removed at intervals and mixed with 8 μl 50 mM EDTA 50% glycerol. The reactions were monitored by agarose gel electrophoresis. Results are shown in Fig 4.

Practical applications of the enzyme

A) M13mp8 SS DNA was used as template for the synthesis of the (−) strand in the presence of an oligonucleotide primer, the four dNTPs, Klenow polymerase and T4 ligase by a standard protocol (14). To the polymerisation reaction was added 2 μg of the exonuclease for 30 min at 37°C. The results of this clean-up reaction are shown in Fig. 5.

B) A phosphorothioate oligonucleotide mutagenesis to change the ochre mutant of the β-galactosidase gene fragment in M13mp2 to the wild type sequence by a point mutation was carried out according to the published procedure (14) with the exception that the nitrocellulose filtration step was replaced by addition of 2 μg of the T5 exonuclease for 30 min at 37°C. The mutational efficiency was determined by the ratio of blue vs. colourless plaques after transfection.

Sequence analysis

DNA sequence data was obtained from the Genbank data bank (15), translations and comparisons were performed using the Genejockey program (Biosoft, Cambridge, UK).

RESULTS

Overexpression of T5 exonuclease from pJONEX44 construct

This construct overproduced T5 exonuclease to the same extent as the pJON45 construct previously reported by us (8) despite the anticipated higher copy number of the new pUC based system and shorter untranslated upstream region. Yield of purified exonuclease was approximately 3 mg per g of cell paste.

Demonstration of single-strand specific endonuclease

The results in Table 1 show that mixtures of double and single-stranded DNAs (each of different phenotype) give rise to plaques

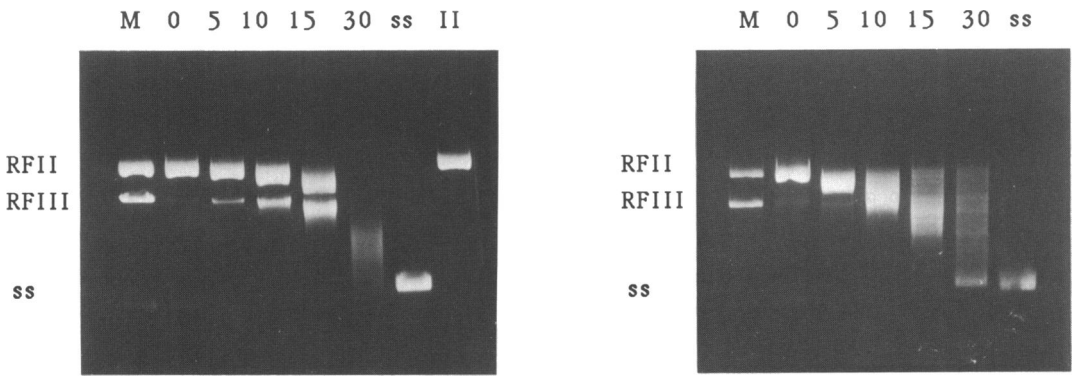

Fig. 4. Time-course of the reaction of T5 exonuclease with nicked DNA in the presence of 10 mM Mg^{2+} (left) and 0.1 mM Mg^{2+} (right). Lane M, marker of nicked (RFII) and linear (RFIII) DNA. Lane II, starting material, nicked DNA. Lane ss, single-stranded circular DNA. Lanes 0−30 show the reaction products at 0−30 min. Conditions as described in Materials and Methods.

of either blue or white phenotype when transfected into competent cells. However, when these DNA mixtures are exposed to T5 exonuclease before transfection only the phenotype derived from the double-stranded DNA is recovered in transfected cells. Thus, the presence of a single-strand specific nuclease activity is indicated by these results.

The results shown in Figure 2 demonstrate that under conditions where linear double-stranded, and circular single-stranded DNA are degraded, double-stranded circular DNA (RFIV) remains largely intact (lanes 3 and 4 in Fig 2). These results confirm the biological assay results.

Action of T5 exonuclease on nicked DNA

The influence of Mg^{2+} concentration on the reaction of T5 exonuclease with nicked (RFII) DNA is shown in Fig 3. At Mg^{2+} concentrations of 10 and 1 mM the nicked DNA is totally degraded (lanes 1 and 2). At a concentration of 0.1 mM (lane 3) a band migrating with single-stranded M13 can clearly be seen, indicating that complete gapping of the nicked DNA has occured.

The results presented in Fig 4 show the influence of Mg^{2+} concentration on the time-course of the reaction. A band with electrophoretic mobility intermediate between that of RFII (nicked) and RFIII (linear) is clearly visible after 5 and 10 min. with 10 mM Mg^{2+} in the assay (Fig 4, left). The gel also shows extensive linearisation (after 10 and 15 min.) and almost total digestion of the substrate DNA after 30 min. Thus, the nicked DNA (RFII) is partially gapped by the exonucleolytic activity of the enzyme before this species is attacked by the endonuclease activity. The substrate DNA now has two free 5'-ends and can be totally degraded by the exonuclease.

The action of T5 exonuclease on RFII DNA in the presence of 0.1 mM Mg^{2+} followed a different mechanism (Fig 4, right). The nicked DNA under these conditions could be completely gapped to yield a product migrating close to a single-stranded circular marker. The results show that there is little linearisation of the substrate under these conditions compared with the reaction at higher magnesium concentration. Evidence for the single stranded circular nature of the gapped DNA is based on the fact that it can be repolymerized in the presence of DNA polymerase I, the four dNTPs and T4 ligase to RFIV DNA.

The usefulness of the enzyme is shown in Fig. 5 where in a mixture of RFIV and RFII M13mp2 DNA, as produced by a polymerisation reaction using the SS DNA as template, the latter is completely removed by incubation with the enzyme. The RFII DNA is digested from the unligated 5'-ends by the exonuclease to produce a single stranded gap which in turn is subject to hydrolysis by the endonuclease.

The combination of these activities has further proved advantageous in the widely used phosphorothioate oligonucleotide mutagenesis method (14). As in any other mutagenesis method, destruction of the wild type SS DNA used as the template is of paramount importance. In the past this was achieved by filtration of the polymerisation reaction over nitrocellulose which retains SS DNA. However, because of losses during the filtration this procedure requires somewhat larger amounts of DNA which are not always available. Replacing this filtration step by incubation with the exonuclease does not only remove RFII DNA as shown in Fig. 5, but also very efficiently the wild type SS DNA as shown by a mutational efficiency of routinely 90%. This represents a considerable improvement in efficiency in addition to the smaller amounts of DNA required.

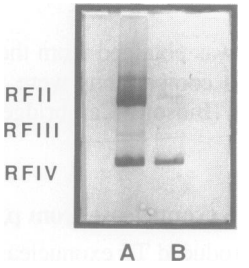

Fig. 5. Gel electrophoretic analysis of a polymerisation reaction using SS M13 mp8 DNA as template and Klenow polymerase as detailed in the Experimental Section. A, Polymerisation reaction without treatment with T5 exonuclease; B, the same reaction after treatment with the exonuclease.

Sequence comparisons

The N-termini of the DNA polymerases from *Thermus aquaticus* (Ta Pol), *Escherichia coli*. (Ec PolI) and *Strepto-*

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T5 Exo    19  RRNLMIVDGTNLGFRKHNN    T5 Exo   125  RGVEADDMAAYIVKLI
Sp polA   15  KKKLLLIDGSSVAFRAFFA    Sp PolA  123  AQYEADDIIGTLDKLA
Ec PolI    6  QNPLILVDGSSYLYRAYHA    Ec PolI  109  SGVEADDVIGTLARKA
Ta pol    11  KGRVLLVDGHHLAYRTFHA    Ta Pol   115  PGYEADDLASLAKKAK

CONSENSUS     ---L-LVDG-----R---A    CONSENSUS     -G-EADD-------K--

T5 Exo   147  LISTDGDWDTLLT          T5 Exo   197  AIMGDLGDNIRGVEGIGAKRG
Sp PolA  144  TIVS-GDKDLIQL          Sp PolA  198  ALMGDKSDNIPGVTKVGEKTG
Ec PolI  130  LIST-GDKDMAQL          Ec PolI  180  ALMGDSSDNIPGVPGVGEKTA
Ta Pol   136  RILT-ADKDLYQL          Ta Pol   187  ALTGDESDNLPGVKGIGEKTA

CONSENSUS     -I-T-GDKD--QL          CONSENSUS     ALMGD-SDNIPGV-G-GEKT-
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Fig. 6. Homologous regions of the N-termini of DNA polymerases from *Thermus aquaticus* (Ta Pol), *Escherichia coli*. (Ec PolI) and *Streptococcus pneumoniae* (Sp PolA) and T5 exonuclease (T5 Exo). Consensus sequences are shown based on the presence of a particular residue in at least three of the four sequences.

Table I. Plaque assay for single-strand specific endonuclease activity

DNA Mixture	T5 Exo Treated	Number of Plaques Blue	Colourless
RFIV M13mp2TAA ssDNA M13mp18	NO	150	160
RFIV M13mp2TAA ssDNA M13mp18	YES	0	365
RFIV M13mp18 ssDNA M13mp2TAA	NO	402	190
RFIV M13mp18 ssDNA M13mp2TAA	YES	375	0
RFIV M13mp18*	NO	543	0
RFIV M13mp2TAA*	NO	0	460
ssDNA M13mp2TAA*	NO	0	590
ssDNA M13mp18*	NO	735	0

* Approximately 8 ng of each pure DNA was used to transfect competent SMH50 cells.

coccus.pneumoniae (Sp PolA), share four regions of conserved amino acid sequence with T5 exonuclease (T5 Exo). These results and a consensus sequence are shown in Fig. 6. Consensus sequences are shown based on the presence of a particular residue in at least three of the four sequences.

DISCUSSION

The T5 exonuclease overproducing plasmid pJONEX44 described here is very similar to the plasmid pJON45 which we described previously (8). However, in pJONEX44 the λPL is approximately 90 bps upstream of the D15 start codon, in the old construct pJON45, this distance is about 280 bps. Furthermore, the new construct pJONEX44 is based on the high copy number, well characterised pUC19 (16) vector, whereas the old construct is a pDOC55 derivative, which was in turn constructed from pBR322 (12). The new pUC-based vector also has a larger number of unique restriction enzyme recognition sites compared with pDOC55. The new construct also retains the excellent antisense repression system present in pDOC55. Thus, any low level transcription from the repressed λP_L is repressed by transcription from the convergent lac promotor as described by O'Connor and Timmis (12). This is of particular importance when the cloned gene product is expected to be toxic, e.g. in the case of a nuclease. Upon heat induction the stronger λP_L directs high level expression of the cloned gene. Levels of exonuclease produced by the two plasmids were indistinguishable (data not shown), despite the expected higher copy number of our new pUC19 based construct. Upon induction of exonuclease the cells ceased growing after one or two doublings, indicating that toxicity of the nuclease, rather than amount of synthesized mRNA, is the limiting step in the overproduction of this particular protein.

Overexpressed phage T5 D15 exonuclease repeatedly co-purified with low levels of a single-strand specific endonuclease. Extensive efforts were made to free the exonuclease of endonuclease activity by various methods described in the Matrials and Methods section. They were all unsuccessful. The enzyme preparations lacked completely any double-strand specific endonuclease as testified to by the biological and agarose gel assay results presented here.

The action of the enzyme on nicked DNA depended upon the conditions employed in the reaction. At Mg^{2+} concentrations of 1−10 mM the nicked DNA substrate was partially gapped allowing the endonuclease to act on the exposed single-stranded regon. This is shown by the presence of a band running between RFII and linear DNA (RFIII). This result demonstrates that a nick alone is not a substrate for the endonuclease as suggested by Moyer and Rothe (6), rather, the nick is first exonucleolytically gapped and the newly created single-stranded region is subject to endonucleolytic cleavage. We show that this endonuclease activity can be suppressed at low Mg^{2+} concentrations.

Analysis and comparison of several DNA polymerase amino acid N-terminus sequences with the T5 exonuclease sequence revealed four conserved regions. The enzymes *E. coli* DNA polymerase I (Ec PolI) (17) and Ta Pol (18) have been shown to possess 5′−3′ exonuclease activity. Sequence similarities have been noted for Ec Pol I and Ta Pol (19), for Pol I and T5 exo (7, 8) and for *S. pneumoniae* polymerase A (Sp PolA) and Ec PolI (20). The regions conserved between the T5 exo and polymerases known to possess a 5′−3′ exonuclease are good candidates for regions involved exonuclease activity. The invariant residues within these consensus sequences are attractive targets for substitution in site-directed mutagenesis experiments in order to determine the active site of the cloned T5 exonuclease. The consensus sequences all occur within similar regions of the primary structure of the enzymes. When residues of similar chemical character are taken into account similarities between the four consensus regions become even more apparent.

We have detected a single-stranded endonuclease which co-purifies with T5 exonuclease under a variety of chromatographic procedures. The endonuclease activity can be effectively suppressed by using low concentrations of Mg^{2+}. The enzyme preparation lacked endonuclease activity on double-stranded circular DNA. The ability of the enzyme to digest linear single- and double-stranded DNA coupled with the ability to suppress the endonuclease activity may prove useful. The enzymes distributive mode of action and 5′−3′ directionality make it an ideal complement to exonuclease III, a 3′−5′ exonuclease. As the examples given indicate, the enzyme could have application in purifying double-stranded closed circular DNA from nicked, linear, or single-stranded contaminants prepared for use in site-directed mutagenesis protocols (14, 21−23), or for the purification of plasmid DNA from sheared genomic DNA contaminants. The enzyme could also be used in a destructive test system for putative single-stranded vectors as double-stranded circular DNA vectors would be resistant to the enzyme whilst single-stranded, or linear DNA would be destroyed.

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