On the Activities of *Escherichia coli* Exonuclease III

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Exonuclease III (Exo III) of *Escherichia coli* is a DNA-modifying enzyme very frequently used in molecular biology. The experiments described here were carried out with the aims of reliably controlling exonuclease activity and of learning more about the enzyme's specificities. The dependence of Exo III activity on factors such as temperature (including heat inactivation), the concentrations of Exo III and NaCl, and the concentration and shape of 3' termini was investigated. Double stranded DNA was found to be a competitive inhibitor of the enzyme activity. Some four nucleotide 3' protrusions were shown to be sensitive to Exo III digestion. The synchronism of deletion was also examined. Implications for the proposed mechanism of activity are discussed. © 1993 Academic Press, Inc.

Although all evidence indicates that Exonuclease III (Exo III)[2] of the bacterium *Escherichia coli* (1) acts within the cell as a repair endonuclease at apurinic and apyrimidinic (AP) sites rather than as an exonuclease (2–4), its exonuclease activity is widely being used for various DNA manipulations *in vitro*: exonucleolytic digestion of double strand DNA from its 3' end generates a single-stranded template, which provides a substrate for labeling reactions (5), for instance, or the enzymatic determination of the sequence by resynthesis of the second strand (6–8). Furthermore, after incorporation of α-thiophosphate nucleotides Exo III itself can be used for fragment termination in an enzymatic sequencing reaction (9,10). Treatment of the single strand tail with a mutagen, such as bisulfit (11), or the hybridization of mismatched oligonucleotides (e.g., 12) result in a mutagenesis of specific regions. In combination with single strand specific nucleases, like S1 nuclease from *Aspergillus oryzae*, Exo III introduces deletions either at nicks randomly created with DNase I (13) or more directed at restriction sites. This is used for generating gaps (e.g., 14,15) or positioning restriction fragments for maximising the expression of a cloned gene (16). The positions of genetic markers (17) and repetitive sequences (18) have been mapped this way. Using deletion clones, protein binding sites have been localized either by a rapid filter assay (8) or by steric blockade of the Exo III passage (e.g., 19), and binding proteins have been identified (20). The production of a set of stepwise deleted clones (21–23) simplifies DNA characterization and provides an ordered approach toward the sequencing of longer DNA stretches.

Exo III is the product of the *x*th gene of *E. coli* which has been completely sequenced (4). The enzyme is a monomeric, globular protein of 31 kDa. It is a double strand specific 3'-5' exonuclease, an exonucleolytic RNase H, a phosphomonoesterase at 3' ends (1,24), as well as an endonuclease at AP sites (3) and urea-*N*-glycosides (25). All these activities are thought to be catalyzed by a single active site (26,3).

The work presented here set out from the desire to control reliably the exonuclease activity of Exo III and to learn more about the enzyme's specificities. To this end, various factors influencing the reaction were examined. From the data generated, it was possible to look in more detail at the catalytic functioning of Exo III. The conclusions drawn on the molecular mechanism of the enzyme's activity are being discussed.

MATERIALS AND METHODS

DNA preparation. Plasmid and cosmid DNA was prepared by alkaline lysis as described in detail elsewhere (27). The DNA concentration and the percentage of covalently closed circular (ccc) DNA in the preparation were determined by the increased fluorescence of DNA-intercalated ethidium (27). Only preparations

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[2] Abbreviations used: Exo III, Exonuclease III; AP, apyrimidinic; ccc, covalently closed circular; TAK, Tris/acetate/potassium; nt, nucleotide.

with more than 95% ccc DNA were used. All restriction digestion was carried out in a single Tris/acetate/potassium (TAK) buffer (28). After cleavage the DNA concentration and the degree of linearization were again measured by the fluorescence assay and confirmed by gel electrophoresis. Cleavage was called complete if more than 97% of the DNA was cut. When the DNA was double digested, it was cut to completion with the enzyme producing 3′ protrusions before the other enzyme was added.

Deletion with Exo III. Deletion with Exo III and S1 nuclease was carried out in a one-tube reaction (22). Linear DNA (1.5 μg) was incubated with 180 units (U) Exo III in 10 μl 15 mM Tris–HCl (pH 8.0), 0.66 mM $MgCl_2$. The sample was equilibrated to the relevant temperature before Exo III was added. Portions of 1.8 μl were removed from the sample in intervals of usually 1 min and immediately mixed with 3.6 μl water which had been preheated to 100°C. The samples were kept at 68°C for 5 min to assure the complete denaturation of the enzyme. After cooling to room temperature, 7.2 μl of 20 mM Na-acetate, pH 4.6, 0.5 M NaCl, 2 mM $ZnSO_4$, 10% glycerol, and 2.4 μl S1 nuclease (4.5 U/μl) were added. Subsequent to a 10-min incubation at room temperature, the S1 nuclease was inactivated upon a pH shift by adding 3 μl of 0.8 M Tris–HCl, pH 8.2, 80 mM $MgCl_2$ and 20 mM EDTA. Eight microliters of each aliquot was run on agarose gels. For the examination of the activity of Exo III, several parameters of these standard conditions were varied as indicated under Results. To obtain circular deletion clones, the remaining 10 μl were incubated with 1 μl of Klenow enzyme (0.25 U) at 37°C for 2 min, after which 0.9 μl of all nucleotide triphosphates (0.25 mM each) was added for 15 min at 37°C to create blunt ends. Ligation was carried out with 5.7 μl ligase buffer (80 mM Tris–HCl, pH 7.5, 30 mM dithiothreitol, 20 mM $MgCl_2$, 3 mM spermidine), 0.9 μl 10 mM ATP, and 2 μl T4 DNA ligase (2 U; BRL) at 12°C overnight. Competent cells were transformed, and DNA from randomly selected colonies was isolated.

Gel electrophoresis. In order to determine the rate of Exo III digestion, the DNA products of deletions were run at 5 V/cm in gels of 0.6 to 1% agarose in 40 mM Tris-acetate, pH 8.2, 1 mM EDTA (e.g., Fig. 1). By comparison to standards, fragment sizes were calculated on the assumption that, within a certain size-range, the mobility of the DNA is inversely proportional to its length (29). For each chosen size range (0.8 to 2.9 kb; 1.4 to 4.9 kb; 3.9 to 9.1 kb) at least four marker molecules were used. Mobility measurements were taken into account only when the calculated length of each marker did not differ from its actual size by more than 2% (e.g., ±60 bp for 2.9 kb) and the calculated variation in length did not exceed 0.7% of the upper limit of each size range (e.g., ±20 bp for fragments between 0.8 and 2.9 kb).
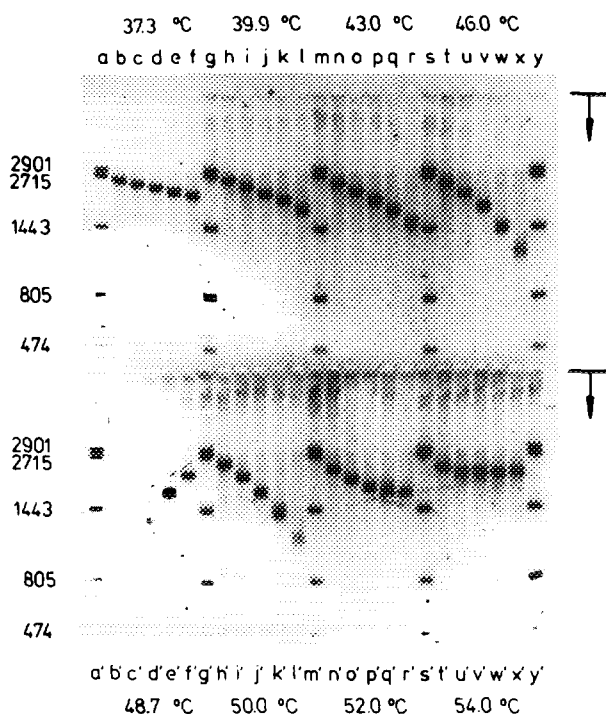

FIG. 1. Rate determination of Exo III deletions at different temperatures. Plasmid pUC18 (32) was cut with *PstI* + *Bam*HI and digested with Exo III under standard conditions. Aliquots from each reaction were taken in 1-min intervals. Run on an ethidium-stained agarose gel, the fragment sizes were determined by comparison to a DNA standard (sizes given on the margin). The samples from the reaction at 48.7°C were loaded in reverse order (f′ to b′).

RESULTS

Temperature dependence of Exo III. The activity of Exo III is very dependent on the incubation temperature (Figs. 1 and 2). Between 22 and 46°C, it doubles every 6°C. The data shown in Fig. 2 were actually accumulated over a period of more than 2 years with various DNA substrates and eight different lots of enzyme, illustrating the high degree of reproducibility. Variation of the temperature allows good control of the exonuclease activity. At a saturating enzyme concentration of 120 U Exo III/μg DNA the rate of deletion is about 100 nucleotides/min (nt/min) at 25°C, 200 nt/min at 31°C, 400 nt/min at 37°C, and about 600 nt/min at 41°C. A reduction to 30 U Exo III/μg DNA cuts these rates by half. From Fig. 2, an activation energy E_a of 98.5 kJ/mol was determined, which is in agreement with values published earlier (30,31). The reported transition of E_a was observed at 30°C (30) rather than 25°C (31), although it is statistically not significant for the presented data set accumulated from many different experiments.

Above about 46°C, the heat inactivation of the enzyme becomes apparent. The rate is reduced exponentially with time. From incubations such as those shown in Figs. 1a′–1y′ the time was determined by which the
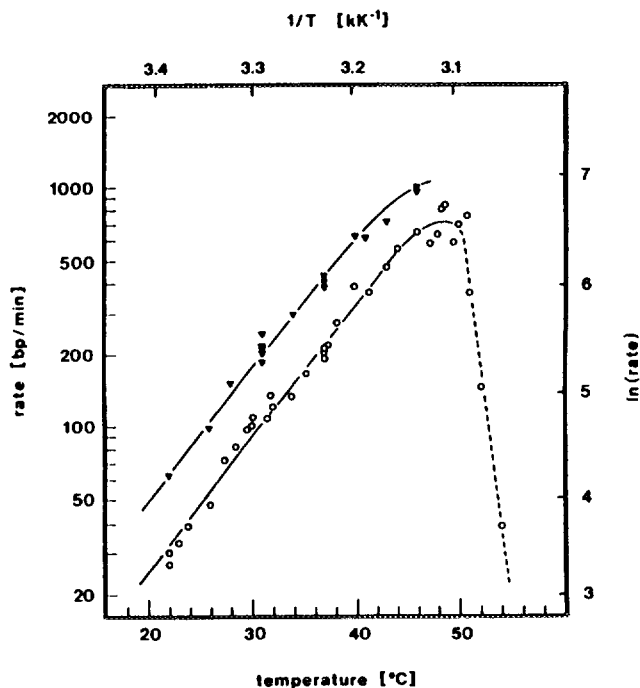
FIG. 2.   Temperature dependence of Exo III. The data were actually accumulated with various substrates over a period of more than 2 years. Deletions were carried out with 30 U enzyme/μg DNA (O) or 120 U/μg DNA (▼). Each point represents the rate at each susceptible DNA terminus averaged from 5 to 15 time points of one reaction. Above 48°C, the averages calculated from 5 intervals are only of limited value, since the rates quickly decreased due to heat inactivation of the Exo III (see Fig. 3).
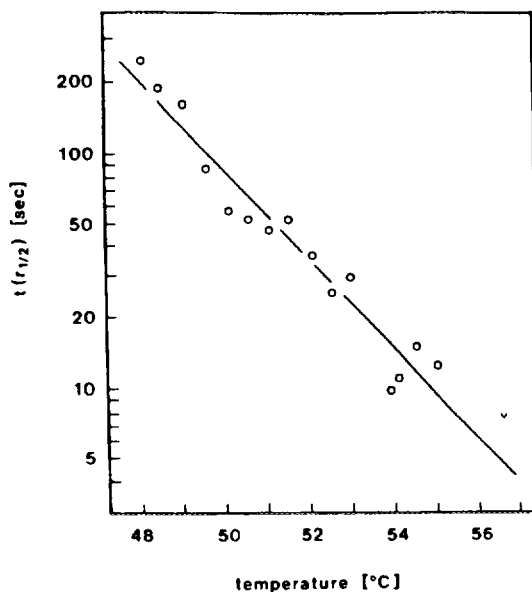

FIG. 3.   Heat inactivation of Exo III. The times were determined during which the rate of deletion was reduced by half. Above 56°C, inactivation was to quick to be followed directly by the gel assay. The arrow at 56.6°C indicates the maximal possible value estimated from the total length of the deletion.

rates dropped by half (Fig. 3). Since these times again get shorter exponentially with increasing temperature, they are equivalent to the half-life of the heat inactivation of Exo III.

Dependence on enzyme and NaCl concentration.

Apart from the incubation temperature, variation of the enzyme concentration can be used for regulating the deletion rate of Exo III (Fig. 4). Using the plasmid pUC18 (2686 bp; Ref. 32) as substrate, saturation is reached at an about 20-fold molar excess of Exo III to plasmid (75 U/μg DNA). At an enzyme concentration of 120 U/μg DNA the rates at 41, 37, and 31°C are in the ratio of 3/2/1. The ratio changes to 2.7/2/1 at very high saturation (600 U/μg DNA).

Increasing the NaCl concentration linearly decreases the activity of Exo III over the whole range of enzyme concentration (Fig. 5). There is no enzymatic activity detectable above 300 mM NaCl. Using almost identical incubation conditions, results have been reported suggesting a complete inactivation of the Exo III above 145 mM NaCl (33). Deletions were carried out on numerous DNAs with five commercial lots of Exo III supplied by two manufacturers and with enzyme isolated in the laboratory from an overproducing strain in order to rule out

differences due to either the batch of enzyme or the DNA substrates used. In all cases, Exo III exhibited the response to NaCl concentration shown in Fig. 5.

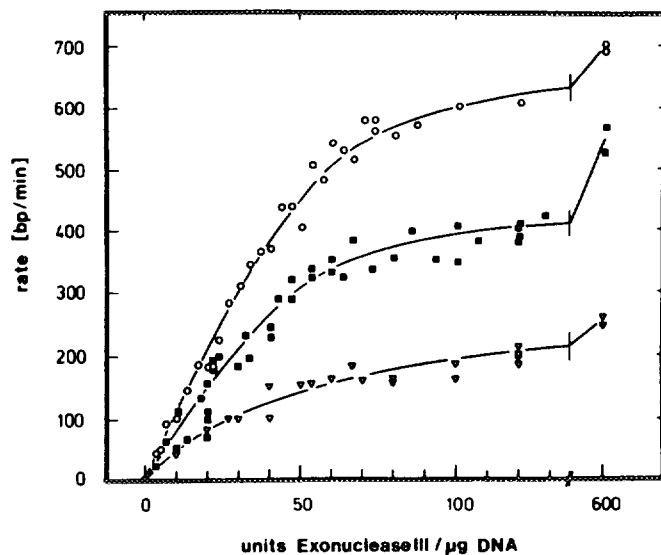*Influence of 3' ends and DNA concentration.*   The amount of Exo III used in deletion experiments is gener-

FIG. 4.   Dependence of the rate of Exo III digestion on enzyme concentration. In a standard reaction of 15 μl, linear plasmid DNA was incubated with increasing concentrations of Exo III at 31°C (▽), 37°C (■), and 41°C (O).
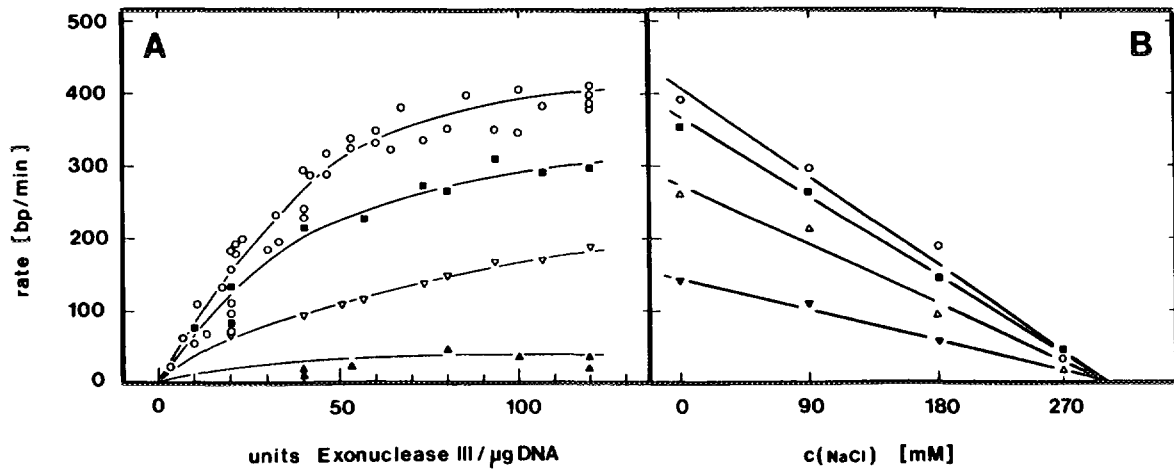
FIG. 5. Dependence of the activity of Exo III on the NaCl concentration. A, Plasmid pUC18 was linearised with *Pst*I + *Xba*I and unidirectionally digested at different Exo III concentrations in presence of 0 mM (O), 90 mM (■), 180 mM (∇), and 270 mM NaCl (▲). B, The average rates at 120 U (O), 80 U (■), 40 U (△), and 20 U (▼) Exo III/μg DNA are shown. The regression lines meet on the axis at 300 mM (± 5 mM) NaCl.

ally given in units per picomole susceptible 3' end, which would be sensible only if uncut DNA and resistant ends exert no influence on Exo III activity. To examine the effect of the concentration of ends and of the shape of the DNA termini, the plasmid pAH9 (5450 bp; unpublished result) was utilized as an indicator molecule. It was linearized by a double digest with *Xba*I and *Pst*I, whose unique hexamer sites are separated by 6 bp, and was subsequently purified. *Xba*I produces a 4 nt 5' overhang, which is digested by Exo III, while the 4 nt 3'-protruding ends created by *Pst*I are not a substrate. So, the linear pAH9 DNA is unidirectionally digested by Exo III. In order to test the effect of concentration of ends on activity at fixed total DNA concentration, 375 ng (104 fmol) of such DNA were mixed with 1125 ng (585 fmol) of another plasmid, pJH1 (2913 bp; Ref. 8), which was added either uncut (Fig. 6: ccc), or after being linearized with either *Xba*I or *Pst*I (Fig. 6). The three different mixtures were deleted under standard conditions (see Materials and Methods) but with only 20 U Exo III/μg DNA. At this nonsaturating enzyme concentration, any effect on account of the concentration of the DNA termini or their shape would be indicated by an immediate change in the rate of deletion (Fig. 4). No such differences were seen. Deletion rates were the same (overall range 1.28-fold) whether the added DNA was linear (138 nM ends) or ccc (21 nM; 6.6-fold less) and whether added linear DNA had sensitive or resistant ends (150-fold difference in ratio of sensitive/resistant ends). Additional controls containing 1500 ng of pAH9 or pJH1 *Pst*I + *Xba*I digests gave the same result. Similar results were obtained also with indicator DNAs smaller than pAH9, which allowed an even higher precision in the determination of the deletion rates (not shown). In all cases, the deletion rates were found to be independent of the concentration and type of the DNA termini.

In order to determine the apparent K_m and maximal velocity V of the Exo III reaction, which follows Michaelis–Menten kinetics, 187, 375, 750, and 1500 ng of *Pst*I + *Xba*I linearized pUC18 DNA were incubated with 30 U Exo III. The apparent K_m and the maximal velocity V were determined from direct-linear plots (Fig. 7A) and Eadie–Hofstee diagrams (e.g., Fig. 7B) of the observed rates. At 31 and 37°C the K_m was 50 μM base pairs, while at 41°C the K_m increased to 75 μM base pairs. The respective maximal velocities V at 31, 37, and 41°C were found to be 50, 115, and 220 pmol of released nucleotides per minute. The same measurements were
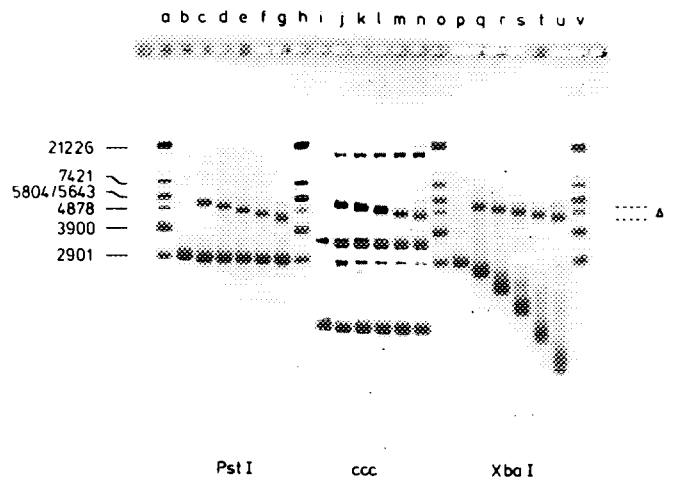

FIG. 6. Independence of the Exo III on the concentration and type of 3' ends. *Pst*I + *Xba*I cut pAH9 DNA (5450 bp) was mixed with plasmid pJH1 (2913 bp) and digested with Exo III at a nonsaturating enzyme concentration. The pJH1 DNA had been cut with either *Pst*I (b, c–g) or *Xba*I (p, q–u) or had been added as ccc DNA (i, j–u). The deletion rates were almost identical in all three reactions. The sizes of the standard molecules in lanes a, h, o, v are given on the margin.
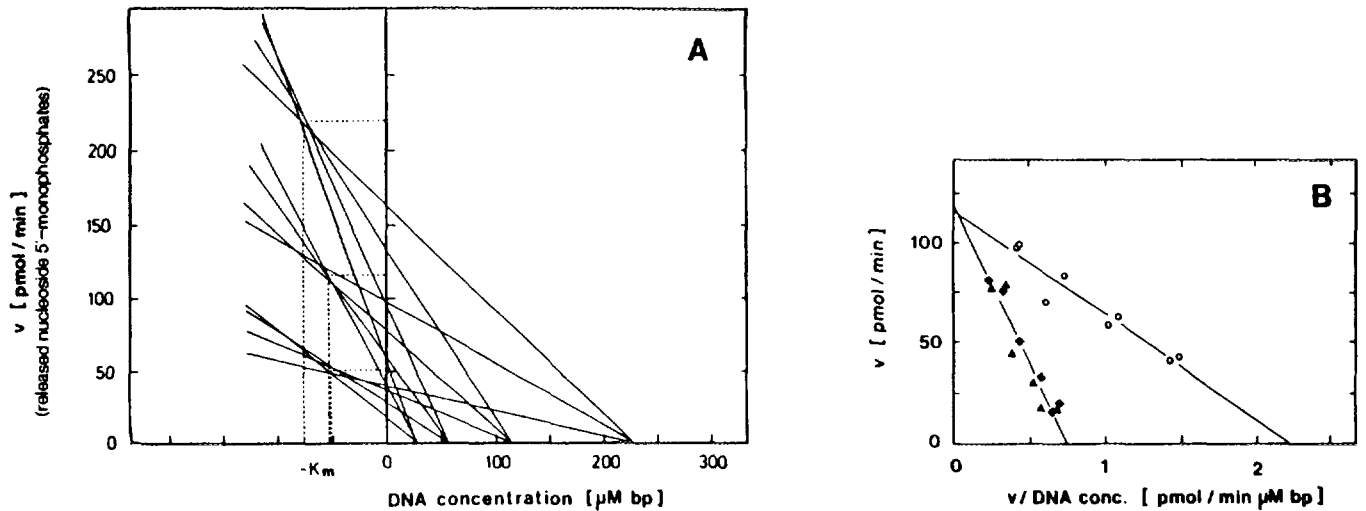
FIG. 7. A, Determination of the apparent K_m and maximal velocity V of Exo III. pUC18 was linearised with $PstI$ + $XbaI$. 187, 375, 750, and 1500 ng DNA were digested with 30 U Exo III in 10 μl at 31, 37, and 41°C. From the DNA concentrations and the rates of deletion the enzyme velocities were calculated. The K_m and V were determined at (top to bottom) 41, 37, and 31°C. B, Determination of the apparent K_I of double stranded DNA. Reactions were carried out at 37°C as described in the legend to A (O) or in presence of additional 750 ng pUC18 DNA (ccc: ♦; $XbaI$ cut: ▲).

carried out in the presence of an additional 750 ng pUC18, uncut or linearized with either $PstI$ or $XbaI$, at 37°C. The added DNA acted as a competitive inhibitor (Fig. 7B) with a K_I of 20 μM base pairs. No difference between the competition with linear or ccc DNA was observed.

Digestion of 4 nt 3'-protrusions. Brutlag and Kornberg (24) found that double stranded polymers with up to three mismatched terminal nucleotides at far recessed 3' ends are hydrolyzed by Exo III as opposed to terminal mismatches of more than 4 nt. Based on this, 4 nt 3' overhangs produced by restriction endonucleases are commonly used to protect DNA termini from Exo III digestion in order to achieve unidirectional deletion (34,21,8).

The 4 nt protrusions produced by $ApaI$ were found to be degraded by Exo III in an experiment with the plasmid pJDH118 (35). Based on this finding, 4 nt 3'-protrusions produced by commercially available restriction enzymes were checked for their resistance to the action of Exo III (Table 1). The plasmids pHDJ18R (36) and pMCS3 (unpublished result) were used. The latter is an extended version of the former, containing 31 unique hexamer sites, 8 unique heptamer or octamer sites for the enzymes $RsrII$, $AscI$, $FseI$, $NotI$, $SgrAI$, $SfiI$, $Sse8387I$, and $SwaI$ and the 18mer recognition sequence of the extremely rarely cutting I-$SceI$ (37) in its polylinker. The termini produced by $AatII$ were checked using the cosmid vector Lawrist-4 (38).

DNA was cleaved with the relevant enzyme and checked for nonspecific degradation of the 3' ends by religation and end-labeling experiments. Incubation

with Exo III was carried out as described under Materials and Methods but with 160 and 280 U/μg DNA. Prior to the addition of the enzyme and after an incubation time of (usually) 1, 3, and 30 min, aliquots were removed, heat-inactivated, and treated with S1 nuclease. Most DNA termini were not substrates for Exo III (Table 1), but fragments created by $ApaI$, $BanII$, $Bsp1286I$, $HaeII$, and $KpnI$ were degraded (Fig. 8 and Table 1). However, only the small fragments adjacent to the $ApaI$ site were digested in DNA cut with $BanII$ (not shown). Similarly, the two rates of disappearance of the $Bsp1286I$ fragments (Fig. 8i) indicate a selective digestion of ends causing unidirectional deletion in some fragments and bi-directional digestion in others. In all cases, only 4 nt-protrusions with dCyt at the very 3' position were found to be degraded by Exo III. Furthermore, the ends produced by $HaeII$ and $KpnI$, with only a single dCyt in the terminal position, required a higher enzyme concentration and longer incubation to achieve a degree of degradation similar to that at ends with two adjacent dCyt nucleotides. This sequence specificity corresponds with the fact that in double stranded DNA dCyt is known to be a preferred substrate for Exo III (39). One alternative possibility would be that these ends become sensitive by annealing to like ends, producing a nicked double-strand structure. This is ruled out, because quantitative data of nearest neighbour analysis (40) predicts that $NsiI$, $PstI$, $SphI$, or $SstI$ end duplexes (resistant ends) are more stable than reannealed $KpnI$ (sensitive) ends.

The distinct bands of deleted DNA visible in the gel indicate an initial burst of activity followed by a slower

Enzyme	End	Substrate	Activity
AatII	GAC̲GT C	Lawrist-4	−
ApaI	GGGC̲C̲ C	pHDJ18R	++
BanII	GRGC̲Y̲ C	pHDJ18R	±±
Bsp1286I	GDGC̲H̲ C	pHDJ18R	±±
HaeII	RGC̲GC̲ Y	pHDJ18R	+
HgiAI	GWGC̲W C	pHDJ18R	−
KpnI	GGTAC̲ C	pHDJ18R	+
NlaIII	NC̲ATG N	pHDJ18R	−
NsiI	ATGC̲A T	pMCS3	−
PstI	CTGC̲A G	pHDJ18R	−
I-SceI	GATAA C	pMCS3	−
	GTTAT C	pMCS3	−
SphI	GC̲ATG C	pHDJ18R	−
SstI	GAGC̲T C	pHDJ18R	−

Note. The activity of Exo III was tested on all 4 nt 3′-protrusions produced by commercially available restriction endonucleases (see text). dCyt nucleotides in the overhang are underlined.

and more constant rate of hydrolysis. A similar effect was observed by Donelson and Wu (41), although at 5°C and far lower Exo III concentrations. Digestion of the same intensity (rate × time = constant at a fixed enzyme concentration) at susceptible 4 nt 3′-protrusions produced deletions of identical length, but a higher overall degree of degradation was obtained at low temperature (e.g., 0.5, 1, 20 min at 41°C versus 5, 10, 200 min at 22°C; Figs. 8c and 8d), suggesting an effect of the thermal breathing of the DNA ends.

Synchronism of Exo III. An even rate of deletion within a population of molecules can be a crucial experimental factor, for example in the preparation of nested deletion clones. To examine the synchronism of Exo III deletions, plasmid DNA was unidirectionally deleted from blunt ends or 4 nt 5′-protrusions. A 50- to 75-fold molar excess of Exo III was used in order to minimize an influence of the nonprocessivity (42) and sequence specificity (39) of the enzyme. The DNA was then recircularized and transformed into *E. coli* cells. The plasmid DNA of individual, randomly selected clones was isolated and sized by gel electrophoresis (28). To check on the unidirectionality of the deletion, the plasmid DNA

of all clones obtained from a transformation (more than 2000) was isolated and cut with *Hind*III, whose site is located 13 bp behind the *Pst*I 3′-end used for strand protection. In all cases, more than 95% of the DNA could be linearized.

The distribution of the plasmid sizes in each deletion approximates a Poisson distribution (e.g., Fig. 9, top). The standard deviation serves as a good description for the synchronism of the Exo III reaction. It increases linearly with the length of deletion (Fig. 9, bottom). For the calculation of the regression line in Fig. 9, the origin of the graph was not taken into account. The standard deviation of a mere 4 bp which can be extrapolated for zero deletion confirms the relevance and accuracy of the measurements. From the regression line, the increase in standard deviation was determined to be 22 bp per 100 bp of Exo III deletion. However, about 9% of the sized plasmids were found to be disproportionally far from the mean thereby distorting the figure given above. The increasingly wide distribution of this small percentage of the clones was found to be produced during the Exo III digestion, though, rather than by any other manipulation involved, such as the S1 nuclease treatment, for
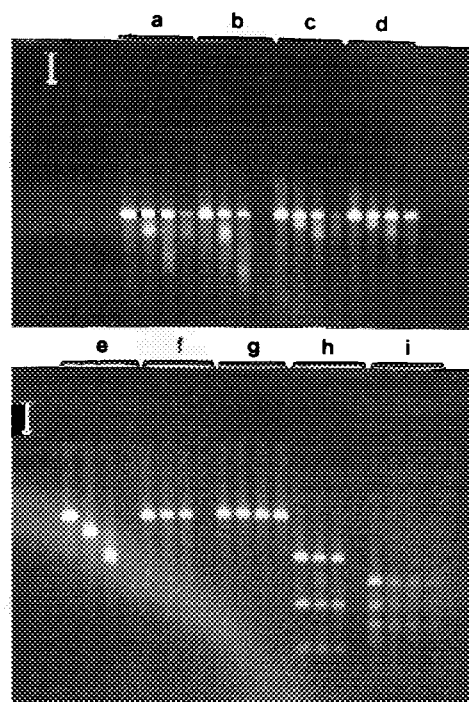

FIG. 8. Degradation of 4 nt 3′-protrusions. *Apa*I-cut DNA was digested with (a) 160 U or (b) 280 U Exo III/μg DNA at 37°C for 0, 1, 3, and 30 min (left to right). The same DNA was incubated with 160 U/μg DNA for 0, 5, 10, 200 min at 22°C (c) and 0, 0.5, 1, and 20 min at 41°C (d). As a control (e), *Hind*III-linearized plasmid was digested as in (a). f, g, and h are incubations of DNA cut with *Kpn*I, *Sst*I, and *Hae*II, respectively, with 280 U Exo III/μg DNA at 37°C for 0, 1, 3, and 90 min. Plasmid cut with *Bsp*1286I (i) was digested with Exo III as in (a).
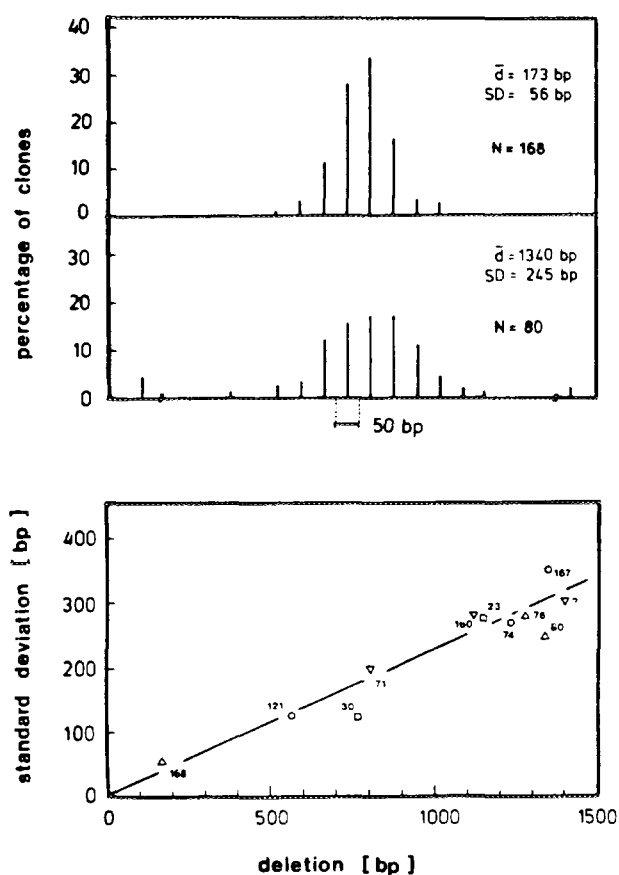
FIG. 9. Synchronism of Exo III. (Top) Size distribution of individual plasmids after Exo III deletion. The percentage of plasmids within size ranges of 50 bp are shown. The average length of deletion (d), the standard deviation (SD), and the total number of clones (N) are indicated. (Bottom) Increase of the standard deviation of the size distribution with increasing length of deletion. Each symbol represents a set of deletion clones as shown in the top. The number of clones sized for each set is given. Digestion was started at 4 nt recessed ($\triangle$) or blunt ($\bigcirc$) DNA termini. Data from two other sources are also included ($\square$: 30; $\triangledown$: 43). The correlation of the regression line is 0.94.

instance (data not shown). With only the remaining 91% of the clones taken into account, the standard deviation is much reduced to about 7.5%.

For many applications, as the isolation of nested deletion clones for sequencing, it is interesting to know how many clones have to be picked and analyzed in order to obtain a full range of deletion clones. This number can be calculated from the total standard deviation of an Exo III deletion and the hence known clone distribution (e.g., Fig. 10), information instrumental, for example, in decisions on the strategy for sequencing a DNA stretch. The fraction of clones falling into a certain size range around the mean of deletion is represented by the percentage of area under the graph of the distribution that is limited on either side of its mean by the given size

constraint. The relevant values can be found in any statistical table (e.g., 44). With a limit of ±100 bp as in Fig. 10, for example, 95.5% of the clones would fall into this region at a standard deviation of 50 bp (half the given variation of ±100 bp), and in a reaction in which the standard deviation would be 100 bp 68.3% of the clones could be expected within a range of ±100 bp.

DISCUSSION

The activities of Exo III permit a wide variety of applications in DNA modification. Regulation by variation of the incubation temperature generates highly reproducible results. To obtain reliable deletion rates, however, the determination of the DNA concentration is a prerequisite, due to the competitive inhibition of the Exo III by double stranded DNA. On the other hand, the frequency of cleavage of the restriction enzyme producing the DNA termini is irrelevant. The apparent K_m of 0.05 mM base pairs for the exonuclease activity is about 35-fold less than the 1.75 mM reported for the enzyme's AP-endonuclease activity (25).

Heat-inactivation of the Exo III at 70°C as described here instantly terminates the digestion ($t[r_{1/2}] < 20$ ms → ~1 nt). The low buffer concentration used in the reaction is rather convenient, since after the heat-inactivation the incubation conditions can easily be changed to meet the requirements of subsequent DNA manipulations, such as the restriction digestion of various deletion aliquots for mapping purposes (unpublished results).

Weiss has proposed that all the functions of Exo III are catalyzed by a single active site (26,3). According to his common-site model, the enzyme contains three regions. Figure 11 shows this model fitted to the helical structure of DNA and extended on the basis of the data presented in this manuscript as discussed below. One region (a) recognizes a deoxyribose on the strand opposite the strand that is cleaved, while a second (b) recognizes a space created by a missing or displaced base. Only then, the active site (c) cleaves the phosphoester bond.

A refinement of this model reported by Kow (31), based on experiments with various substituent-N-glycosides, is contradictory in itself. For the AP endonuclease activity of Exo III, it proposes that only the presence of a secondary amine at the N-glycosylic linkage facilitates a ring opening of the (deoxy)ribose thereby producing a change in the stereochemistry which results in the interstrand space necessary. The inability to cleave at substituents with a tertiary amine is explained by the fact that there would be an unstable iminium intermediate. For the exonuclease activity, however, the stability of such iminium intermediates would be required, since the N-glycosylic bond in nucleotides is present as a tertiary amine. Apart from the fact that one secondary
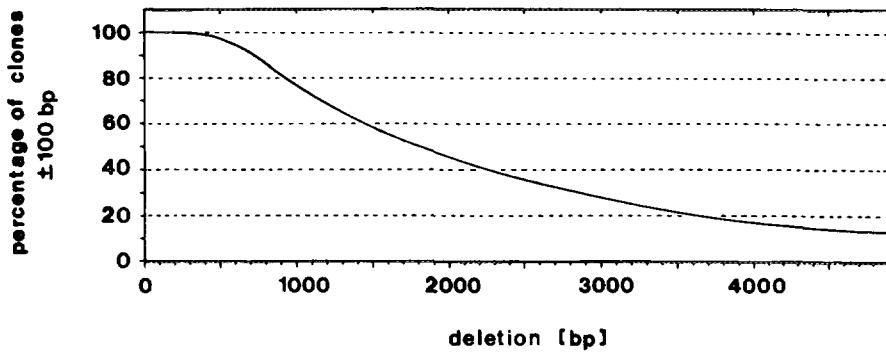
FIG. 10. Calculation of the percentage of clones (from any taken aliquot) falling into a size range of ±100 bp around the mean of deletions of up to 5 kb.

amine used was not a substrate for Exo III, the model not only implies the catalysis of two different mechanisms at the single active site of the enzyme, but more variations would actually be required for its activities as an AP endonuclease in absence of any amine or as a 3′ phosphomonoesterase with neither amine nor glycoside present. This seems rather unlikely, especially since the endonuclease and the exonuclease activity are concomitantly lost by the introduction of point mutations in the *x*th gene (45,3). Additionally, given a 3′-phosphoryl terminus on DNA, Exo III releases the inorganic phosphate before proceeding as an exonuclease (1), although the monoesterified group is removed at a lower rate than the phosphodiester linkage (3), a fact not explained by the proposed mechanism.

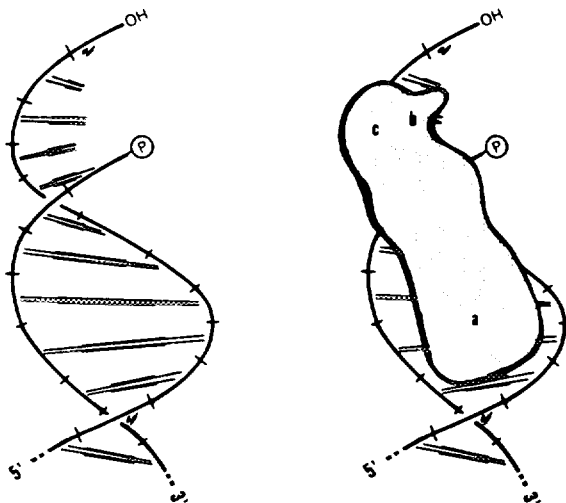Fitted to a double helical DNA structure, Fig. 11 extends the model of Weiss according to the following find-

FIG. 11. Schematic model of the complex formed between Exo III and a DNA terminus with a 4 nt 3′-protrusion. The free DNA (left) and the complex are shown. a, b, and c indicate the three regions of the enzyme (see text).

ings: The enzyme is double-strand specific, but 3′ protrusions of up to 4 nt can be digested, although with lower efficiency. There is a temperature dependence of the degradation at 4 nt 3′-protrusions suggesting, together with the higher K_m at 41°C, that the degree of double strand breathing at the terminus influences the ability of the enzyme to reach the 3′-most phosphodiester bond. Considering the enzyme's Stokes radius of 2.29 nm (26), these data suggest a binding of region (a) to the major groove. Since the substrate is located 3′ to the binding site by about 5 bp, the model allows for an attachment from one side of the DNA. The resulting close contact to the sugar-phosphate backbone of the undigested strand could explain the deoxyribose specificity of Exo III. Any 3′ overhang longer than 4 nt cannot be reached by the active site for the lack of a double strand opposite to it.

No data are available whether the enzyme–DNA complex influences the DNA structure. However, Exo III, highly processive below 20°C, becomes distributive at higher temperatures coinciding with a change in its activation energy. This suggests that the association and dissociation with the DNA is not rate limiting above 30°C (31). Also, binding of enzymes to nonspecific sequences does not need to influence the DNA conformation (46,47).

Exo III requires an interstrand space created by the absence or the displacement of a base for its activity. The space itself rather than some feature of the unpaired base seems to be recognized (3). Exo III cannot even initiate hydrolysis at a single strand break at 5°C and 70 mM salt, conditions that favor base stacking across the nick (41). However, 3′-protruding nucleotides are not endonucleolytically released, although no such restraint is present, nor is any nucleotide exonucleolytically removed before the 3′-terminal phosphoryl group is released. All this points to a critical connection between the C3′ phosphate and the base. When the base at the C1′ position of the sugar would be displaced to provide the necessary space, the phosphate at the C3′ posi-

tion would concomitantly pushed toward the enzyme. Thus, either the protein–DNA complex would be destabilized or the displacement of the base would be inhibited. The model of a merely steric inhibition of the enzyme's activity as long as both the base and the 3′ phosphoryl group are present is consistent with all the data available.

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