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A label-free assay of exonuclease activity using a pyrosequencing technique

Karl-Heinz Gührs [a,*], Marco Groth [b], Frank Grosse [a]

[a] Biochemistry Workgroup, Leibniz Institute for Age Research–Fritz Lipmann Institute, D-07745 Jena, Germany
[b] Genome Analyses Workgroup, Leibniz Institute for Age Research–Fritz Lipmann Institute, D-07745 Jena, Germany

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ABSTRACT

Enzymes with 3′–5′ exonuclease activities are important in promoting the accuracy of DNA replication and DNA repair by proofreading. The alteration of the function of these enzymes by endogenous or exogenous effectors could, therefore, have a considerable impact on DNA replication and ultimately on genome integrity. We have developed a label-free high-throughput screening method for quantifying the effects of different reagents on exonuclease activity. The assay is based on a hairpin-forming biotinylated oligonucleotide substrate that contains one or more exonuclease-resistant phosphorothioate nucleotides. The activity and specificity of the selected 3′–5′ exonuclease is determined indirectly using a sensitive pyrosequencing reaction after cleanup of the samples. In this pyrosequencing step, the amount of nucleotides filled into each position of the exonucleolytically degraded 3′ end of the substrate can be recorded quantitatively and equals the amount of the nucleotides removed by the exonuclease. This system allows the estimation of both processivity and efficiency of the exonuclease activity. We have employed compounds reported in the literature to inhibit the exonuclease activities of either exonuclease III or the large fragment of polymerase I (Klenow fragment) to evaluate the assay.

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Exonucleases are DNA-degrading enzymes that play important roles in a variety of physiological and cellular processes. The double-stranded DNA-specific 3′–5′ exonucleases (35dsExos)[1] are a small group within this enzyme family, whose other members are involved mostly in the processing of RNA or DNA–RNA hybrids. The major human 35dsExo is the protein TREX1 that forms a homodimer with two catalytic sites [1,2]. The functions of this enzyme and the related TREX2 protein are still not completely clear, but their inactivation is connected with impaired apoptosis and autoimmune-like inflammatory diseases [3,4].

In contrast to the autonomous TREX proteins, the majority of 35dsExo activities are embedded in multifunctional proteins or protein complexes. The existence of 35dsExo domains or subunits is generally considered to be of crucial significance for certain DNA polymerases and DNA helicases to ensure accuracy in DNA replication, repair, and recombination [5–7]. It was shown that the modulation of the internal 35dsExo activity of DNA polymerases by mutation resulted in significant losses of accuracy [8–11]. DNA integrity is also maintained by DNA-binding complexes with 35dsExo subunits such as Rad1 and Rad9 (9–1–1 complex) [12,13] or Mre11 (MRN complex) [14–17], which have essential functions in DNA repair and recombination.

In addition, there are some proteins that possess 35dsExo activities with uncertain physiological roles. Ape1 and Ape2 are endonucleases in DNA repair processes with specificity for apurinic and apyrimidinic sites. Their detected 35dsExo activities point to additional roles in replicative processes [18,19]. The endonuclease Fen1, which removes displaced single-stranded DNA segments, also has 35dsExo activity that is complementary to the respective function of polymerase delta [9,20]. Last but not least, the tumor suppressor protein p53 was reported to possess 35dsExo activity [21–25].

Taking into account their general impact on the maintenance of cellular DNA, the determination of the actual contributions of particular 35dsExos would help to further clarify the mechanisms safeguarding genetic stability. At this moment, however, there are no substances that efficiently inhibit 35dsExos. This lack of 35dsExo inhibitors in general, and of specific inhibitors of individual 35dsExos in particular, is caused partly by the absence of a useful high-throughput assay. Currently, radioactively labeled substrates and densitometric analyses of the autoradiograms are used to study 35dsExos, and this is laborious and requires high costs per sample. Besides the need for special safety equipment and waste disposal, such methods permit analysis of only a limited number of samples. Fluorometric techniques have been used in many high-throughput enzyme assay systems, but there is only one fluorescence-based method reported that has been used to screen for

* Corresponding author. Fax: +49 3641 656288.
E-mail address: kguehrs@fli-leibniz.de (K.-H. Gührs).

[1] *Abbreviations used:* 35dsExo, double-stranded DNA-specific 3′–5′ exonuclease; ExoIII, exonuclease III; KF, Klenow fragment; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin.

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inhibitors of the 35dsExo activity of Ape1 [26]. Instead, fluorometric analyses have been adapted mainly to kinetic analyses of exonucleases [27–31].

Here we present a label-free assay for the *in vitro* determination of 35dsExo activity. This assay exploits the high sensitivity and high-throughput potential of an established pyrosequencing protocol. Pyrosequencing couples the accuracy of a DNA polymerase and the sensitivity of bioluminescence detection [32] and is used for genetic analyses such as the identification of single-nucleotide polymorphisms and DNA modifications [33]. Our assay design allows the semiquantitative study of the effects of modulators of 35dsExo activity. To demonstrate the feasibility of our assay, we have estimated the ranges of both applicable substrate amounts and detectable enzyme activities for the model enzymes exonuclease III (ExoIII) and the Klenow fragment (KF) of polymerase I of *Escherichia coli*.

Materials and methods

Materials

The exonuclease substrate ExoSub1 (Fig. 1) was designed in-house and synthesized by MWG Biotech (Ebersberg, Germany). The deoxyribooligonucleotide has the sequence 5′-CACTAGTCaCTCCAGCAGCTTCAACGGTTT∗TTCCGTTGAAGCTGCTGGAGtGACTAGTG-3′. The lowercase letters represent phosphorothioate moieties, and the asterisk marks a biotinylated nucleotide. The underlined residues are complementary and form the double-stranded part of the substrate. The substrate was designed in such a way that both activity and sequence preference of a 3′–5′ exonuclease can be read simultaneously from one reaction. For efficient removal of components of the exonuclease reaction that might interfere with the pyrosequencing reaction, a biotin group was attached to the oligonucleotide at synthesis. This "handle" was positioned at maximal distance to the reaction center of the exonuclease reaction to reduce any interference with enzyme function. The incorporation of phosphorothioate groups in the stem of the hairpin structure near the 3′ end prevents the destabilization of the backbone due to excessive degradation of the double strand. The sequence of the 3′ terminus covers all nucleotides to monitor substrate specificities of the 35dsExos. If the exonuclease displays unrestrained activity, up to seven nucleotides can be removed, leaving a protruding 5′ end. The 3′ end of the remaining double-stranded segment acts as a primer in the following pyrosequencing step, in which the matching nucleotide is incorporated by a DNA polymerase. The second phosphorothioate bond near the 5′ end was included to minimize substrate degradation by possible 5′–3′ exonuclease contaminations of the protein preparations. The double-stranded part of the hairpin also covers a recognition site of the restriction endonuclease SpeI that can be used to provide a uniform substrate with recessed 3′ ends for the identification of material not digested by the exonucleases.

E. coli ExoIII (150,000 U/mg) and KF of *E. coli* polymerase I (20,000 U/mg) were purchased from New England Biolabs (Frankfurt, Germany). The 10-fold concentrated stock solution of buffer Y, which was used as the reaction buffer, was supplied by Fermentas (St. Leon-Rot, Germany). Buffer substances, NaF, dimethyl sulfoxide (DMSO), captan, and pifithrin (2-(2-imino-4,5,6,7-tetrahydrobenzothiazol-3-yl)-1-*p*-tolylethanone hydrobromide) were purchased from Sigma–Aldrich (Deisenhofen, Germany). Streptavidin Sepharose was obtained from GE Healthcare (Munich, Germany), and the Pyro Gold Reagent Kit was supplied by Biotage (Uppsala, Sweden). The inhibitor CRT0044876 (7-nitroindole-2-carboxylic acid) was obtained from Calbiochem (Merck Chemicals, Nottingham, UK). Mirin (6-(4-hydroxyphenyl)-2-thioxo-2,3-dihydro-4(1H)-pyrimidinone) was supplied by Hit2Lead (ChemBridge, San Diego, CA, USA). All aqueous solutions were prepared using ultrapure water produced by a Milli-Q device (Millipore, Schwalbach, Germany).

Exonuclease reaction

The substrate oligonucleotide ExoSub1 was dissolved in TE buffer (10 mM Tris–HCl and 1 mM EDTA [ethylenediaminetetraacetic acid], pH 7.5) to yield a final concentration of 100 μM, and the solution was aliquoted and kept at −20 °C. Prior to use, the substrate was diluted in buffer Y (33 mM Tris–acetate, 10 mM magnesium acetate, 66 mM potassium acetate, and 0.1 mg/ml bovine serum albumin [BSA], pH 7.9) to a final concentration of 250 nM (0.25 pmol/μl). This solution was heated to 95 °C and cooled to room temperature within 4 h to ensure the correct annealing of the hairpin structure. The obtained substrate stock solution was used to prepare the working solution by further dilution with RB buffer immediately before use. Aliquots of 8 μl of the working solutions were placed in microcentrifuge tubes, each of which had been previously filled with 34.5 μl of buffer Y.

The stock solutions of the inhibitors were prepared from solid materials by dissolving weighed amounts in DMSO to concentrations of 20 mM each and further diluted with DMSO as required. The enzyme solutions were prepared from the supplied stock solutions by dilution in buffer Y to the desired concentrations.

The reaction vessels containing the substrate solution were provided with 2.5 μl of inhibitor solution or DMSO. The reactions were started by the addition of 5 μl of enzyme solution and incubated for either 2 min at room temperature (ExoIII) or 15 min at 30 °C (KF). Then the enzymes were inactivated by incubation at 75 °C for 10 min. The samples were stored at −20 °C until the pyrosequencing assay.

Pyrosequencing technique

Biotin-labeled ExoSub1 samples were immobilized on streptavidin Sepharose and purified using the PyroMark Vacuum Prep Tool (Biotage) as described by Groth and coworkers [34]. Immobilization, washing, renaturation, and pyrosequencing of labeled oligonucleotides were performed in 96-well plates. Buffers used for these steps were purchased from Biotage. Briefly, the purified immobilized oligonucleotides were treated with washing buffer

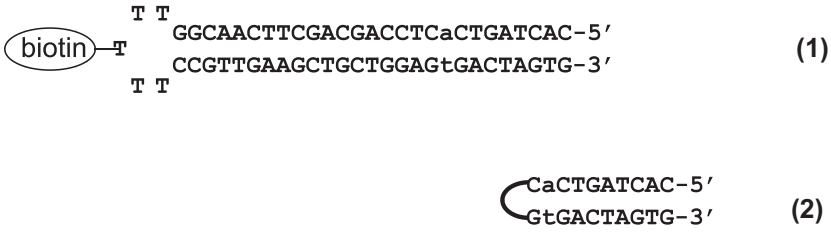

Fig. 1. Structure of the hairpin deoxyribooligonucleotide substrate ExoSub1 (1). Lowercase letters represent nucleotides that form phosphorothioate bonds at their 3′ ends, which resist the attack of 3′–5′ exonucleases. The complicated structure (1) is symbolized in further drawings by formula (2).

and transferred to 40 μl of annealing buffer (Biotage). To prevent aberrant strand hybridization after the washing steps, the solution was heated to 80 °C and subsequently cooled at a rate of 0.2 °C/s to 20 °C in a Peltier Cycler PTC-200 (MJ Research, Waltham, MA, USA). The sequencing reaction was performed using the Pyro Gold Reagent Kit in a PSQ 96MA pyrosequencing instrument (Biotage) using the sequence analysis mode SQA (sequence analysis) according to the manufacturer's instructions. SQA means that four cycles of the addition of deoxynucleoside triphosphates were performed. Each cycle comprised the addition of four dNTPs in the dispensation order ACGT. Dispensed deoxynucleoside triphosphates, as well as the enzyme mixture and substrate needed in the pyrosequencing reaction, were purchased from Biotage. The heights of the subsequent signals of the pyrosequencing reaction reflect the amount of the inserted nucleotides, which are equivalent to the percentage of exonucleolytic removal of the particular nucleotide.

Results

To facilitate the understanding of the results, the principle of the assay and the output of the pyrosequencing are shown schematically in Fig. 2. Suitable amounts of substrate as well as detectable enzyme activities were determined using ExoIII and the large fragment of polymerase I (KF). With the preparation protocol described above, the maximal amount of substrate defined by the amount of streptavidin resin used in sample cleanup was determined to be 10 pmol. The use of substrate amounts of 0.8 pmol or more always resulted in reproducible pyrograms that unambiguously reflected the variations of the experimental parameters. The use of substrate amounts of less than 0.4 pmol occasionally failed to give evident pyrosequencing signals, and this might be the consequence of substrate losses during sample cleanup.

As shown in Fig. 3, the sample preparation and pyrosequencing scheme yielded evaluable results when as little as 0.3 pmol of oligonucleotide substrate was treated with suitable amounts of ExoIII. The incubation of 2.00, 0.80, or 0.32 pmol of the substrate with 0.33 U of ExoIII for 2 min consistently brought about the sequence ACTAGTG in the pyrosequencing reaction. This sequence is the result of the fill-in of the 3′ end of the ExoSub1 molecule starting directly downstream of the phosphorothioate-linked nucleotide and, thus, is equivalent to the complete abrasion of the 3′ end by the exonuclease. The observed signal intensities clearly reflected the applied amounts of substrate. To guarantee a reliable interpretation in terms of acceptable signal-to-noise ratios, the substrate amount was fixed at 0.8 pmol in further evaluation assays.

To determine the detectable ranges of enzymatic activities, we prepared serial dilutions of ExoIII and KF and analyzed the sensitivity of the assay in relation to decreasing enzyme amounts. In contrast to ExoIII, there is no scale to estimate the nucleolytic activity of KF. Therefore, we referred to its polymerase activity, which in terms of the proofreading function is related to the 35Exo activity. The results demonstrated that the assay is useful to detect activities of as little as 0.04 U of ExoIII or 35dsExo activity of 0.04 polymerase units of KF under the conditions described in Materials and methods. Using 0.8 pmol of substrate and taking into consideration the specific activities of the enzymes given by the supplier, the studied molar substrate-to-enzyme ratios in the assays ranged from 4 to 90 for ExoIII and from 1 to 27 for KF. Most

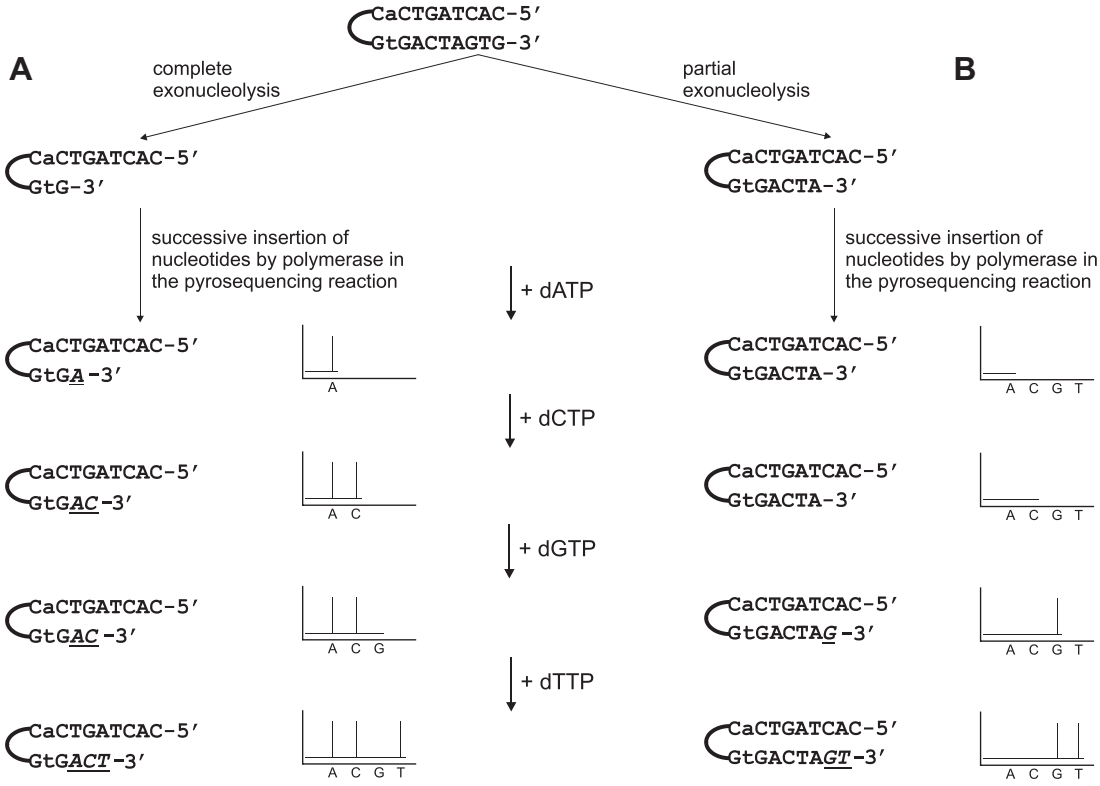

Fig. 2. Schematic illustration of the principal steps of the exonuclease assay. The exonuclease substrate ExoSub1 is shown as a simplified structure introduced by formula (2) in Fig. 1. The 3′ ends of the substrate are converted by a 3′–5′ exonuclease either completely (A) or partially (B), leaving recessed ends of differing lengths. After removal of the exonuclease, the recessed 3′ ends are used as the priming sites for the incorporation of matching nucleotides by a DNA polymerase in the pyrosequencing step. Together with the resulting structures of the termini, the outputs from the first of four pyrosequencing cycles with the dispensing order ACTG of nucleotide triphosphates are presented schematically for a completely recessed 3′ end (A) and for a partially recessed 3′ end (B). Nucleotides added to the 3′ ends in the pyrosequencing steps are displayed as underlined italicized letters.

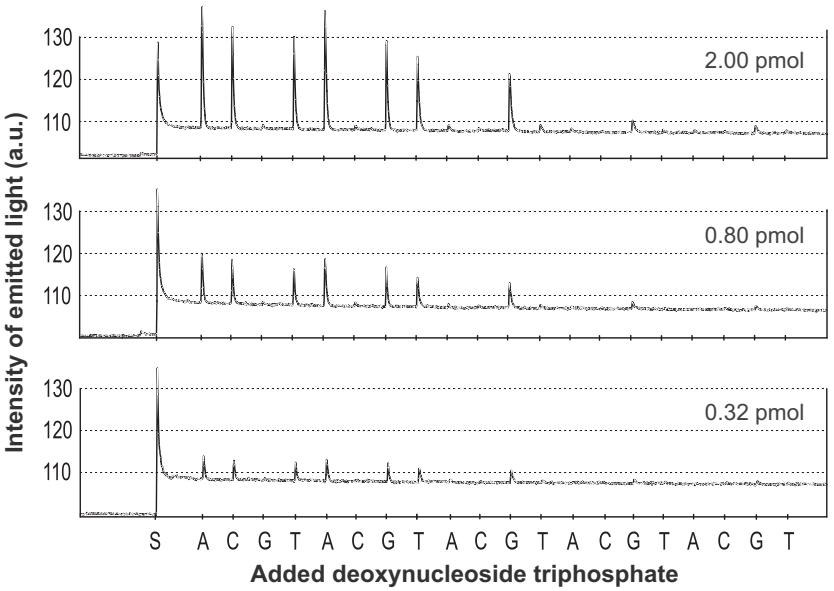

Fig. 3. Pyrograms of ExoIII-treated substrate ExoSub1. Here 2.00, 0.80, or 0.32 pmol of the annealed substrate was digested with 0.33 U of ExoIII for 2 min in a total volume of 50 μl reaction buffer Y. The reaction products were purified and analyzed by pyrosequencing as described in Materials and methods. The uppercase S on the abscissa indicates the injection of substrate needed for the pyrosequencing reaction.

likely, the detection limit of the assay can be further decreased by optimization of the assay conditions in terms of reaction buffer, temperature, and duration of the exonuclease reaction.

Using more than 0.33 U of ExoIII (enzyme-to-substrate ratio > 0.10) always resulted in constant signal intensities of the sequence ACTAGTG in pyrosequencing, indicating the complete removal of the 3′ ends. The reduction of the enzyme amount to less than 0.15 U led to the alteration of the resulting pyrosequencing pattern together with a decrease of signal intensities. Surprisingly, we observed different behavior with KF. Here the result of a reduction of the enzyme amount was predominantly an alteration of the resulting pyrosequencing pattern but not the decrease of signal intensities (Fig. 4). The decrement of the enzyme amount at a given substrate concentration initially increased the intensities of subset pyrosequencing signals, which declined only at further enzyme reduction. In testing a multitude of enzyme-to-substrate ratios, it became evident that the transition from the complete excision to the restricted excision of the 3′ end occurred below an enzyme-to-substrate ratio of approximately 1.0. Presumably, there is a change of the mechanism of exonucleolysis by KF below this value

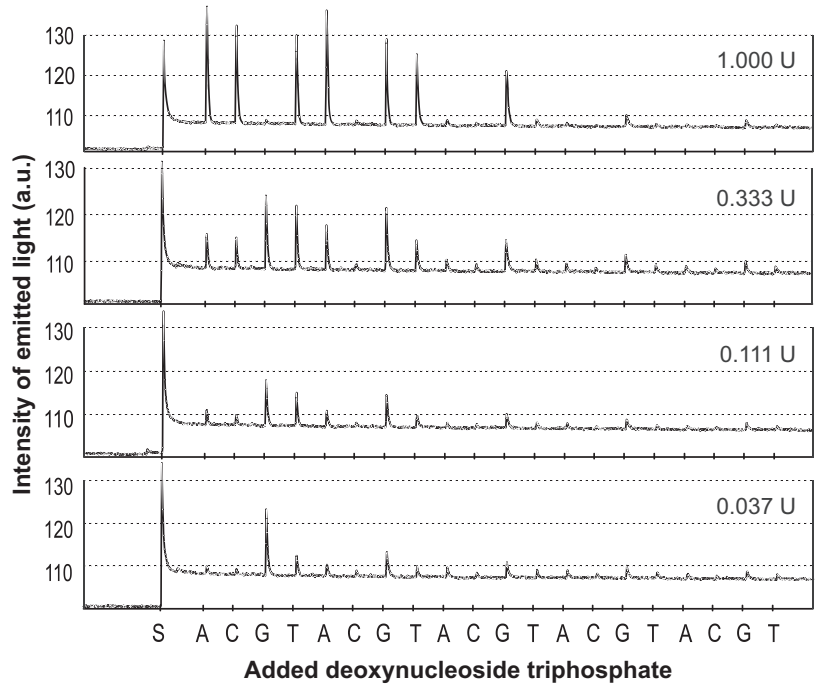

Fig. 4. Pyrograms resulting from treatment of 0.8 pmol of ExoSub1 by decreasing amounts of KF. The annealed substrate was converted by 1.000, 0.333, 0.111, or 0.037 polymerase units of KF at 30 °C for 15 min in a total volume of 50 μl reaction buffer Y. The reaction products were purified and analyzed by pyrosequencing as described in Materials and methods. The uppercase S on the abscissa indicates the injection of substrate needed for the pyrosequencing reaction.

that blocked its progression to completeness. The observations indicated low processivities of both enzymes under the given conditions of slight substrate excess.

To validate the suitability of the assay to measure the inhibition of exonucleases, we applied conditions known to reduce the activities of ExoIII and KF. Inhibition of ExoIII can be achieved by increasing the ionic strength of the reaction buffer [35]. We doubled the concentration of sodium ions to 140 mM by the addition of 75 mM NaCl, leading to the expected reduction of enzyme activity, as shown in Fig. 5. The signal intensities of the sequence ACTAGTG representing complete exonucleolysis were reduced and supplanted, in part, by signals predominantly of the sequence GTG, indicating the presence of partially digested substrate molecules. This observation became more obvious when low amounts of ExoIII were used.

Sodium fluoride [36] and captan [37,38] have previously been reported to inhibit the 35dsExo activity of KF. The effects of these substances are demonstrated in Fig. 6. Under the assay conditions, the presence of 0.1 mM captan had no effect on the 35dsExo activity of KF. In contrast, the 3′–5′ nucleolytic function of this enzyme was clearly suppressed by sodium fluoride at a concentration of 10 mM, as indicated by the predominance of the sequence GTG in pyrosequencing corresponding to incompleteness of substrate conversion.

We also used our assay to test three substances that were previously reported to inhibit individual mammalian 35dsExos. At a concentration of 1 mM, the inhibitors of p53 (pifithrin) and Ape1 (CRT0044876) [26] did not appear to inhibit the 35dsExo activity of either ExoIII or KF. The addition of 1 mM mirin [39], however,

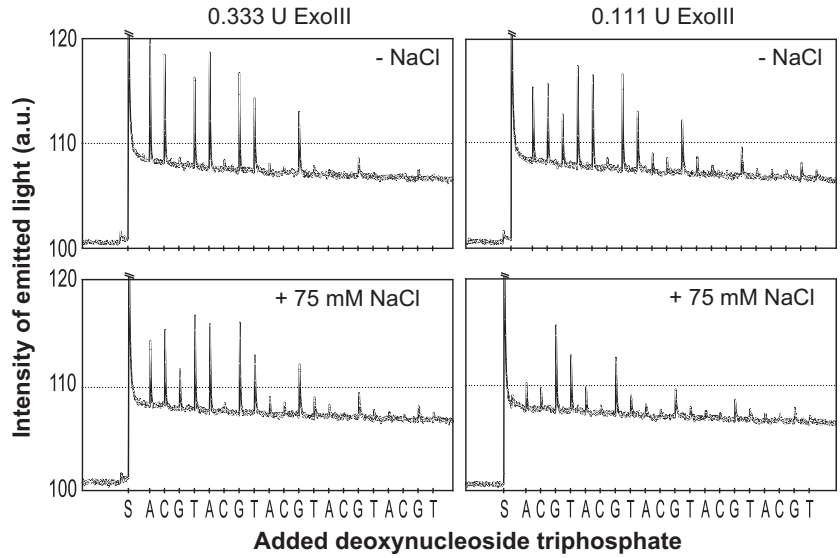

Fig. 5. Inhibition of ExoIII activity by NaCl. The pyrograms of 0.8 pmol of ExoSub1 digested by 0.333 or 0.111 U of ExoIII are shown without (top row) and with the addition of 75 mM NaCl (bottom row) to buffer Y. The reaction products were purified and analyzed by pyrosequencing as described in Materials and methods. The uppercase S on the abscissa indicates the injection of substrate needed for the pyrosequencing reaction.

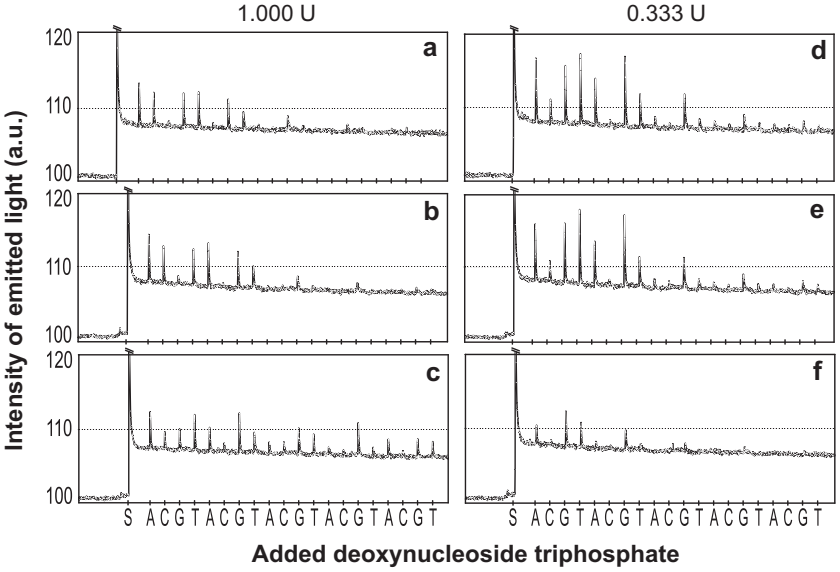

Fig. 6. Effects of 0.1 mM captan (B and E) and 10 mM NaF (C and F) on the exonuclease activity of KF. Results obtained without the addition of interfering substance are shown for comparison (A,D). The pyrograms of 0.8 pmol of ExoSub1 treated with 1.000 U of KF (A–C) or 0.333 U of KF (D–F) are shown. The reaction products were purified and analyzed by pyrosequencing as described in Materials and methods. The uppercase S on the abscissa indicates the injection of substrate needed for the pyrosequencing reaction.

caused a moderate inhibition (⩽50%) of the reaction (Fig. 7). Interestingly, the pyrograms produced by the inhibitory effect of mirin differed from those that were created by a 9-fold reduced amount of the exonucleases. This finding again points to the significance of alterations of the substrate-to-enzyme ratio on the mechanisms of action of both enzymes. The different behavior could also be a consequence of the varying protein contents of the reaction mixtures.

Discussion

Although the principal components safeguarding genome stability have been increasingly elucidated, there are many reports about the participation of additional proteins in fine-tuning or backup of individual steps of these pathways. Some of them are considered directly as alternative 3′–5′ exonucleases, and others might interact with exonuclease complexes to alter their specificity or kinetics. The availability of specific inhibitors would facilitate the evaluation of the influence of such candidate exonucleases on particular cellular processes. The search for inhibitors was one of the goals of the proposed label-free three-stage assay in which the steps of exonuclease reaction and detection by pyrosequencing are concatenated by a purification procedure.

The basis for the quantitative estimation of the exonucleolytic activity is the hairpin structure of the oligonucleotide substrate that ensures a large distance between the reaction center and the biotin moiety used as purification handle. As the results of the experiments using high enzyme activities showed, the two phosphorothioate bonds incorporated into the nucleotide backbone blocked any undesirable destruction of the substrate by the exonucleases. Nevertheless, the 3′ end of the annealed ExoSub1 was used efficiently as a substrate by both enzymes, confirming previous reports about the largely uncompromised structure of phosphorothioate-containing DNA [40,41]. Thus, the phosphorothioate bond near the 3′ end of the oligonucleotide marked the defined endpoint of the complete clearance of the 3′ end. The 3′ hydroxyl group of the remaining G residue was used as the priming site by the polymerase in the pyrosequencing without any noticeable difference compared with phosphodiester substrates. These findings demonstrate that ExoSub1 can be used in the proposed assay without restrictions. There are only two situations that can interfere with the assay. First, the presence of additional DNA distorts the results due to competition with the substrate. Another source of defect is the presence of any substances that compromise the binding of biotin to the streptavidin matrix.

Provided that, first, the annealing of the oligonucleotide ExoSub1 produces only the predicted stable hairpin substrate and, second, the 3′ ends of all substrate molecules are completely abraded by a 35dsExo during the assay, the pyrosequencing must show only signals matching the sequence ACTAGTG resulting from the complete recovery of the 3′ end precedingly degraded by the exonuclease. In addition, the intensities of the pyrosequencing signals, which correspond to the numbers of incorporated nucleotides, must constantly reach the maximal values. These theoretical predictions were indeed observed experimentally when high exonuclease activities were applied (Fig. 3; see also Fig. S1 in the supplementary material). The reproducibility of the results with high or excess activities of exonuclease demonstrated the stability of the substrate and the robustness of the assay. The direct relation between exonuclease activity and signal intensity of the pyrosequencing readout can be used to identify substances that can inhibit the activities of individual exonucleases.

We used the well-investigated 35Exos ExoIII and DNA polymerase I large fragment (KF) to discover the limit of detection of the assay and to set up a general protocol. The presented data show that both exonucleases could be studied below the picomolar range using the reported setup. This very high sensitivity is a prerequisite to studying proteins that cannot be prepared in large quantities, which applies to most of the candidate molecules discussed to contribute to genome maintenance. In addition to the sensitivity, the assay is capable of being used in high-throughput screens given that all steps can be easily automated. This feature is important for the search for inhibitors because the exonucleases of interest have only limited stability under the assay conditions. Therefore, especially the first step of the assay must be performed with maximal speed when screening large substance libraries.

To demonstrate the usability of the assay in inhibitor screening, we tested low-molecular-weight substances that have been

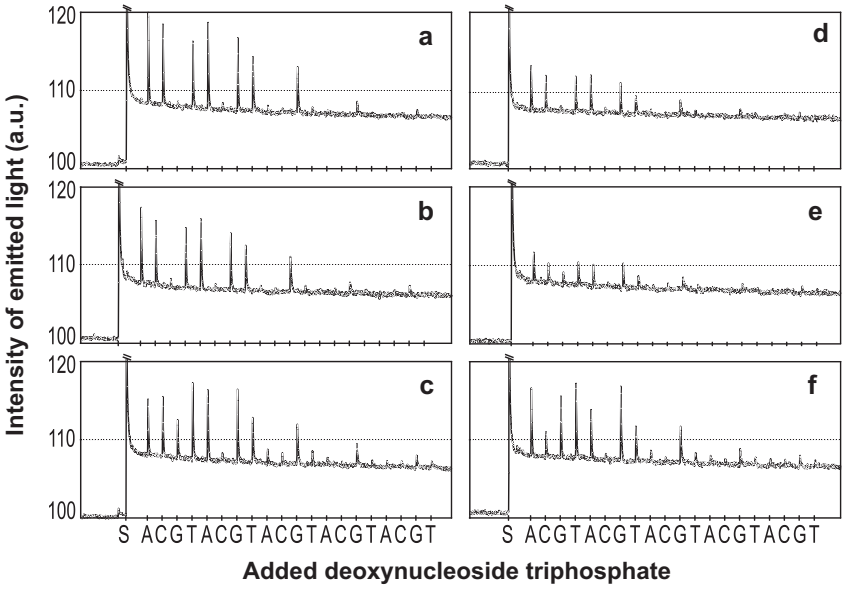

Fig. 7. Effects of mirin on the exonuclease activities of ExoIII and KF. Here 0.8 pmol of ExoSub1 was treated with either 1.000 U of ExoIII (A and B) or 1.000 U of KF (D and E) in the absence (A and D) or presence (B and E) of 1 mM mirin. The pyrograms resulting from the reactions in the absence of mirin with 0.111 U of ExoIII (C) and KF (F) are shown to estimate the efficiency of inhibition by mirin. The reaction products were purified and analyzed by pyrosequencing as described in Materials and methods. The uppercase S on the abscissa indicates the injection of substrate needed for the pyrosequencing reaction.

reported to specifically inhibit individual proteins with 3′–5′ exonuclease activities. Captan, previously reported as an inhibitor of the 3′–5′ activity of Klenow fragment [37,38], was ineffective in our assay (Fig. 6). Also, the p53 inhibitor pifithrin and CRT0044876, proposed to inhibit Ape1 (at 10 μM) and ExoIII (at >50 μM) [26] failed to inhibit the model exonucleases. The only substance that reduced the activities of both 35Exos at a concentration of 1 mM was mirin (Fig. 7), previously shown to inhibit the Mre11 exonuclease at 100 μM [39]. The effect of mirin on KF was more pronounced, although we also observed a certain inhibition of ExoIII in contrast to the original report (Fig. 7). Possibly, mirin can interact with the active center of KF more efficiently than with that of ExoIII, initially supporting the approach to search for specific inhibitors of 35Exos.

The test results using the reference exonucleases demonstrate the importance of selecting an appropriate concentration of the enzyme under study. However, this concentration depends on the purpose of the study. The reduction of the signal intensities of a given sequence in the pyrosequencing step as a consequence of the diminished enzyme activity is required in inhibitor screenings to yield an easily evaluable readout of the assay. On the other hand, the assay can also be used for the investigation of the mechanism of the enzymatic action of 35Exos that rather needs limiting enzyme amounts. This was shown for the determination of the processivity of ExoIII and KF as well as for the investigation of the alteration of enzymatic actions of these enzymes at different substrate-to-enzyme ratios.

The observed alteration of the sequence pattern of the pyrograms at reduced enzyme amounts can be easily explained on the basis of the low processivity of both enzymes. The frequent dissociation of the enzymes leaves recessed DNA ends of different lengths that result in a mixture of species, giving rise to the superposition of various sequences in the pyrosequencing output. The pyrograms, however, demonstrated that the reduction of enzyme amounts did not lead to a random distribution of sequences but rather favored the occurrence of the sequence GTG (Figs. 2 and 3) instead of, or in addition to, ACTAGTG. This might correspond to a preferential dissociation of both exonucleases either after the removal of a guanosine nucleotide or at terminal adenosine residues under conditions of limiting enzyme amount. For ExoIII, this finding is in agreement with the results of Linxweiler and Horz, who showed in an analysis using DNA fragments that ExoIII preferentially stopped after the excision of G residues [42].

In our assay, the processivity of ExoIII at 37 °C was lower than previously reported. Thomas and Oliveira determined the nonprocessive but distributive release of up to 50 nucleotides before ExoIII dissociated from the used poly(dA/dT) double strands [43]. In contrast to our observations, such a high processivity would always remove the complete 3′ end of a bound substrate molecule, giving rise only to the sequence ACTAGTG in pyrosequencing, and the intensities of the signals would be a direct measure of the enzymatic activity. The noticed drop of processivity might be a result of the used enzyme-to-substrate ratios. Whereas previous reports always dealt with the case of excess of enzyme with respect to the DNA termini, the substrate concentration of 16 nM (0.8 pmol/50 μl) in this study exceeded that of ExoIII by factors from 4 to 90. Two models can explain the observed course of the ExoIII reaction. First, the substrate excess can stimulate the rapid dissociation after the release of the guanosine residue known to be a preferred dissociation point. Binding to substrate that had already been degraded during a previous binding event can then result in a completely removed 3′ terminus of the substrate. Second, in a less probable model, the excess of substrate induces extensive DNA binding and, thereby, causes a lack of unbound ExoIII molecules that are required to release bound molecules from the DNA. This would imply a need for collaboration of at least two molecules of ExoIII to drive the reaction along the DNA strand. Some support for this idea is given by the detection of a stable complex of ExoIII with DNA that was observed at 5 °C. The complex was formed at low temperature after the initial removal of 6 nucleotides by the enzyme and turned into an active enzyme with enhanced processivity when the temperature was increased [44].

Because of the lack of known specific exonuclease inhibitors, we tried to mimic inhibition of the activities of ExoIII and KF by application of conditions reported to reduce their activities. This was the only way to verify the assay, although we were aware that the alteration of the enzymatic function by changing the reaction conditions might differ from the situation of actual inhibition of the exonucleases by molecular interactions. The results showed that the assay correctly measured the reduction of exonuclease activities, but the change of the reaction conditions complicated the readout when high salt (ExoIII [Fig. 5]) or sodium fluoride (KF [Fig. 6]) was added to the reaction mixture. High salt concentrations have been described as interfering with proper binding of the essential magnesium ions [45]. The mechanism of fluoride inhibition is progressively understood and is apparently based on the alteration of the binding behavior of the essential magnesium ions in the reactions of many enzymes such as pyrophosphatases [46,47], enolases [48], and ATPases [9,49].

The setup we have presented in this article can be used to search for inhibitors of double-stranded specific 3′–5′ exonucleases by the screening of appropriate substance libraries. Such compounds could be effective antimicrobial agents that act in a novel way without the risk to further facilitate the evolution of resistant pathogens. Alternatively, it provides an opportunity to study the mechanism of individual 3′–5′ exonucleases under a variety of conditions. Benefiting from the small substance requirements, it can also be applied to clarify whether or not a molecule under study actually performs 35Exo activities that can complement or assist the function of the physiological enzymes.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2010.05.019.

References

[1] D.J. Mazur, F.W. Perrino, Identification and expression of the TREX1 and TREX2 cDNA sequences encoding mammalian 3′ → 5′ exonucleases, J. Biol. Chem. 274 (1999) 19655–19660.
[2] M. Brucet, J. Querol-Audi, M. Serra, X. Ramirez-Espain, K. Bertlik, L. Ruiz, J. Lloberas, M.J. Macias, I. Fita, A. Celada, Structure of the dimeric exonuclease TREX1 in complex with DNA displays a proline-rich binding site for WW domains, J. Biol. Chem. 282 (2007) 14547–14557.
[3] M. Morita, G. Stamp, P. Robins, A. Dulic, I. Rosewell, G. Hrivnak, G. Daly, T. Lindahl, D.E. Barnes, Gene-targeted mice lacking the Trex1 (DNase III) 3′ → 5′ DNA exonuclease develop inflammatory myocarditis, Mol. Cell. Biol. 24 (2004) 6719–6727.
[4] D. Kavanagh, D. Spitzer, P.H. Kothari, A. Shaikh, M.K. Liszewski, A. Richards, J.P. Atkinson, New roles for the major human 3′–5′ exonuclease TREX1 in human disease, Cell Cycle 7 (2008) 1718–1725.
[5] L.B. Bloom, X. Chen, D.K. Fygenson, J. Turner, M. O'Donnell, M.F. Goodman, Fidelity of *Escherichia coli* DNA polymerase III holoenzyme: the effects of β, γ complex processivity proteins and ε proofreading exonuclease on nucleotide misincorporation efficiencies, J. Biol. Chem. 272 (1997) 27919–27930.
[6] L. Song, M. Chaudhuri, C.W. Knopf, D.S. Parris, Contribution of the 3′- to 5′-exonuclease activity of herpes simplex virus type 1 DNA polymerase to the fidelity of DNA synthesis, J. Biol. Chem. 279 (2004) 18535–18543.
[7] R.D. Saunders, I. Boubriak, D.J. Clancy, L.S. Cox, Identification and characterization of a *Drosophila* ortholog of WRN exonuclease that is required to maintain genome integrity, Aging Cell 7 (2008) 418–425.
[8] M. Simon, L. Giot, G. Faye, The 3′ to 5′ exonuclease activity located in the DNA polymerase delta subunit of *Saccharomyces cerevisiae* is required for accurate replication, EMBO J. 10 (1991) 2165–2170.
[9] R. Gary, M.S. Park, J.P. Nolan, H.L. Cornelius, O.G. Kozyreva, H.T. Tran, K.S. Lobachev, M.A. Resnick, D.A. Gordenin, A novel role in DNA metabolism for the binding of Fen1/Rad27 to PCNA and implications for genetic risk, Mol. Cell. Biol. 19 (1999) 5373–5382.

[10] Y.T. Hwang, C.B. Hwang, Exonuclease-deficient polymerase mutant of herpes simplex virus type 1 induces altered spectra of mutations, J. Virol. 77 (2003) 2946–2955.
[11] Y.I. Pavlov, S. Maki, H. Maki, T.A. Kunkel, Evidence for interplay among yeast replicative DNA polymerases alpha, delta, and epsilon from studies of exonuclease and polymerase active site mutations, BMC Biol. 2 (2004) 11.
[12] K. Ishikawa, H. Ishii, T. Saito, K. Ichimura, Multiple functions of rad9 for preserving genomic integrity, Curr. Genomics 7 (2006) 477–480.
[13] H.B. Lieberman, Rad9, an evolutionarily conserved gene with multiple functions for preserving genomic integrity, J. Cell. Biochem. 97 (2006) 690–697.
[14] K.M. Trujillo, S.S. Yuan, E.Y. Lee, P. Sung, Nuclease activities in a complex of human recombination and DNA repair factors Rad50, Mre11, and p95, J. Biol. Chem. 273 (1998) 21447–21450.
[15] S. Moreau, J.R. Ferguson, L.S. Symington, The nuclease activity of Mre11 is required for meiosis but not for mating type switching, end joining, or telomere maintenance, Mol. Cell. Biol. 19 (1999) 556–566.
[16] J.H. Lee, R. Ghirlando, V. Bhaskara, M.R. Hoffmeyer, J. Gu, T.T. Paull, Regulation of Mre11/Rad50 by Nbs1: effects on nucleotide-dependent DNA binding and association with ataxia–telangiectasia-like disorder mutant complexes, J. Biol. Chem. 278 (2003) 45171–45181.
[17] R.S. Williams, J.S. Williams, J.A. Tainer, Mre11–Rad50–Nbs1 is a keystone complex connecting DNA repair machinery, double-strand break signaling, and the chromatin template, Biochem. Cell Biol. 85 (2007) 509–520.
[18] M.Z. Hadi, K. Ginalski, L.H. Nguyen, D.M. Wilson 3rd, Determinants in nuclease specificity of Ape1 and Ape2, human homologues of *Escherichia coli* exonuclease III, J. Mol. Biol. 316 (2002) 853–866.
[19] P. Burkovics, V. Szukacsov, I. Unk, L. Haracska, Human Ape2 protein has a 3′–5′ exonuclease activity that acts preferentially on mismatched base pairs, Nucleic Acids Res. 34 (2006) 2508–2515.
[20] Y.H. Jin, R. Obert, P.M. Burgers, T.A. Kunkel, M.A. Resnick, D.A. Gordenin, The 3′ → 5′ exonuclease of DNA polymerase delta can substitute for the 5′ flap endonuclease Rad27/Fen1 in processing Okazaki fragments and preventing genome instability, Proc. Natl. Acad. Sci. USA 98 (2001) 5122–5127.
[21] T. Mummenbrauer, F. Janus, B. Muller, L. Wiesmuller, W. Deppert, F. Grosse, p53 Protein exhibits 3′-to-5′ exonuclease activity, Cell 85 (1996) 1089–1099.
[22] F. Janus, N. Albrechtsen, I. Dornreiter, L. Wiesmuller, F. Grosse, W. Deppert, The dual role model for p53 in maintaining genomic integrity, Cell. Mol. Life Sci. 55 (1999) 12–27.
[23] M. Bakhanashvili, Exonucleolytic proofreading by p53 protein, Eur. J. Biochem. 268 (2001) 2047–2054.
[24] C. Melle, H.P. Nasheuer, Physical and functional interactions of the tumor suppressor protein p53 and DNA polymerase α-primase, Nucleic Acids Res. 30 (2002) 1493–1499.
[25] G. Lilling, N. Elena, Y. Sidi, M. Bakhanashvili, p53-Associated 3′ → 5′ exonuclease activity in nuclear and cytoplasmic compartments of cells, Oncogene 22 (2003) 233–245.
[26] S. Madhusudan, F. Smart, P. Shrimpton, J.L. Parsons, L. Gardiner, S. Houlbrook, D.C. Talbot, T. Hammonds, P.A. Freemont, M.J. Sternberg, G.L. Dianov, I.D. Hickson, Isolation of a small molecule inhibitor of DNA base excision repair, Nucleic Acids Res. 33 (2005) 4711–4724.
[27] L.B. Bloom, M.R. Otto, R. Eritja, L.J. Reha-Krantz, M.F. Goodman, J.M. Beechem, Pre-steady-state kinetic analysis of sequence-dependent nucleotide excision by the 3′-exonuclease activity of bacteriophage T4 DNA polymerase, Biochemistry 33 (1994) 7576–7586.
[28] W.C. Lam, E.J. Van der Schans, C.M. Joyce, D.P. Millar, Effects of mutations on the partitioning of DNA substrates between the polymerase and 3′–5′ exonuclease sites of DNA polymerase I (Klenow fragment), Biochemistry 37 (1998) 1513–1522.
[29] M.R. Otto, L.B. Bloom, M.F. Goodman, J.M. Beechem, Stopped-flow fluorescence study of precatalytic primer strand base-unstacking transitions in the exonuclease cleft of bacteriophage T4 DNA polymerase, Biochemistry 37 (1998) 10156–10163.
[30] H. Kurita, K. Inaishi, K. Torii, M. Urisu, M. Nakano, S. Katsura, A. Mizuno, Real-time direct observation of single-molecule DNA hydrolysis by exonuclease III, J. Biomol. Struct. Dyn. 25 (2008) 473–480.
[31] D. Tleugabulova, L.J. Reha-Krantz, Probing DNA polymerase–DNA interactions: examining the template strand in exonuclease complexes using 2-aminopurine fluorescence and acrylamide quenching, Biochemistry 46 (2007) 6559–6569.
[32] M. Ronaghi, S. Karamohamed, B. Pettersson, M. Uhlen, P. Nyren, Real-time DNA sequencing using detection of pyrophosphate release, Anal. Biochem. 242 (1996) 84–89.
[33] A. Ahmadian, M. Ehn, S. Hober, Pyrosequencing: history, biochemistry, and future, Clin. Chim. Acta 363 (2006) 83–94.
[34] M. Groth, K. Huse, K. Reichwald, S. Taudien, J. Hampe, P. Rosenstiel, G. Birkenmeier, S. Schreiber, M. Platzer, Method for preparing single-stranded DNA templates for pyrosequencing using vector ligation and universal biotinylated primers, Anal. Biochem. 356 (2006) 194–201.
[35] J.D. Hoheisel, On the activities of *Escherichia coli* exonuclease III, Anal. Biochem. 209 (1993) 238–246.
[36] I.A. Potapova, G.A. Nevinsky, V.V. Khomov, O.I. Lavrik, NaF and mononucleotides as inhibitors of 3′–5′ exonuclease activity and stimulators of polymerase activity of *E. coli* DNA polymerase I Klenow fragment, FEBS Lett. 277 (1990) 109–111.
[37] M.J. Freeman-Wittig, R.A. Lewis, Alteration of the exonuclease activities of DNA polymerase I by captan, Biochim. Biophys. Acta 867 (1986) 107–113.
[38] M.J. Freeman-Wittig, W. Welch Jr., R.A. Lewis, Binding of captan to DNA polymerase I from *Escherichia coli* and the concomitant effect on 5′–3′ exonuclease activity, Biochemistry 28 (1989) 2843–2849.
[39] A. Dupre, L. Boyer-Chatenet, R.M. Sattler, A.P. Modi, J.H. Lee, M.L. Nicolette, L. Kopelovich, M. Jasin, R. Baer, T.T. Paull, J. Gautier, A forward chemical genetic screen reveals an inhibitor of the Mre11–Rad50–Nbs1 complex, Nat. Chem. Biol. 4 (2008) 119–125.
[40] S.D. Putney, S.J. Benkovic, P.R. Schimmel, A DNA fragment with an α-phosphorothioate nucleotide at one end is asymmetrically blocked from digestion by exonuclease III and can be replicated in vivo, Proc. Natl. Acad. Sci. USA 78 (1981) 7350–7354.
[41] G. Schreiber, E.M. Koch, W.J. Neubert, Selective protection of in vitro synthesized cDNA against nucleases by incorporation of phosphorothioate analogues, Nucleic Acids Res. 13 (1985) 7663–7672.
[42] W. Linxweiler, W. Horz, Sequence specificity of exonuclease III from *E. coli*, Nucleic Acids Res. 10 (1982) 4845–4859.
[43] K.R. Thomas, B.M. Olivera, Processivity of DNA exonucleases, J. Biol. Chem. 253 (1978) 424–429.
[44] J.E. Donelson, R. Wu, Nucleotide sequence analysis of deoxyribonucleic acid: VII. Characterization of *Escherichia coli* exonuclease 3 activity for possible use in terminal nucleotide sequence analysis of duplex deoxyribonucleic acid, J. Biol. Chem. 247 (1972) 4661–4668.
[45] C.B. Black, J.A. Cowan, Magnesium dependent enzyme in nucleic acid biochemistry and magnesium dependent enzyme, in: J.A. Cowan (Ed.), The Biological Chemistry of Magnesium, VCH, New York, 1995.
[46] A.A. Baykov, A.P. Alexandrov, I.N. Smirnova, A two-step mechanism of fluoride inhibition of rat liver inorganic pyrophosphatase, Arch. Biochem. Biophys. 294 (1992) 238–243.
[47] A.A. Baykov, I.P. Fabrichniy, P. Pohjanjoki, A.B. Zyryanov, R. Lahti, Fluoride effects along the reaction pathway of pyrophosphatase: evidence for a second enzyme pyrophosphate intermediate, Biochemistry 39 (2000) 11939–11947.
[48] J. Qin, G. Chai, J.M. Brewer, L.L. Lovelace, L. Lebioda, Fluoride inhibition of enolase: crystal structure and thermodynamics, Biochemistry 45 (2006) 793–800.
[49] Z. Ahmad, A.E. Senior, Inhibition of the ATPase activity of *Escherichia coli* ATP synthase by magnesium fluoride, FEBS Lett. 580 (2006) 517–520.