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RNA Abasic Sites: Preparation and Trans-Lesion Synthesis by HIV-1 Reverse Transcriptase

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Abasic sites are well-known DNA lesions that occur spontaneously under acidic or oxidative stress, or as intermediates after enzymatic excision of damaged bases.[1] Due to the missing genetic information such sites are highly mutagenic. Therefore, nature has developed a ubiquitous cellular DNA-repair machinery for genome housekeeping.[2] However, cellular RNA lesions are less well known, and, with the exception of a few cases, their biological impact is unclear. For example, RNA abasic sites can occur as a result of the action of RNA *N*-ribohydrolases and can severely affect the vitality of a cell. A famous example of the occurrence of RNA abasic sites is when the peptide toxin, ricin, depurinates a specific adenosine residue on 28S rRNA, which is one of the RNA chains of eukaryotic ribosomes. This leads to poor binding of elongation factors and thus the abortion of protein synthesis. A single molecule of ricin is sufficient to kill a whole cell.[3] Besides this, there is evidence for a new class of RNA-specific lyases in wheat germ that act on rRNA apurinic sites,[4] and, very recently, a repair mechanism for alkylated RNA was found.[5] However, data on the intrinsic chemical mechanism and kinetics of the decay of abasic RNA in comparison to abasic DNA are virtually nonexistent.[6] This prompted us to study the chemistry and chemical biology of RNA abasic sites in vitro in more detail. Here we report preliminary results on the synthesis and translesion-polymerase activity of HIV-1 reverse transcriptase on a RNA template–DNA primer system with an abasic site in the template.

Our synthesis started with the RNA abasic site building block **1** (Scheme 1), the synthesis of which will be described in detail elsewhere. A very similar approach to obtaining abasic RNA was published independently recently;[7] however, only the synthesis but no further biological data was described. Building block **1** contains the photocleavable *R*-1-(2-nitrophenyl)ethyl (1NPE) group at the anomeric center. This group was recently used in oligoribonucleotide synthesis as a 2′-*O* protecting group and has proven superior to the *O*-nitrobenzyl group during photolysis.[8] 2′-*O*-[(Triisopropylsilyl)oxy]methyl (TOM) was chosen as a 2′-*O* protecting group.[9]

To test the performance of this amidite building block in synthesis, we first prepared the RNA 7-mer **2**, which contains

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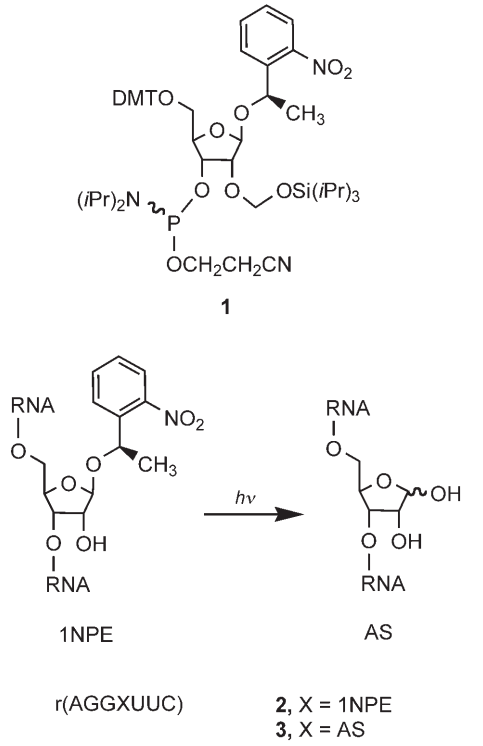

Scheme 1. Chemical structures of the RNA abasic site (AS), its building block (**1**), and the protected intermediate, 1NPE.

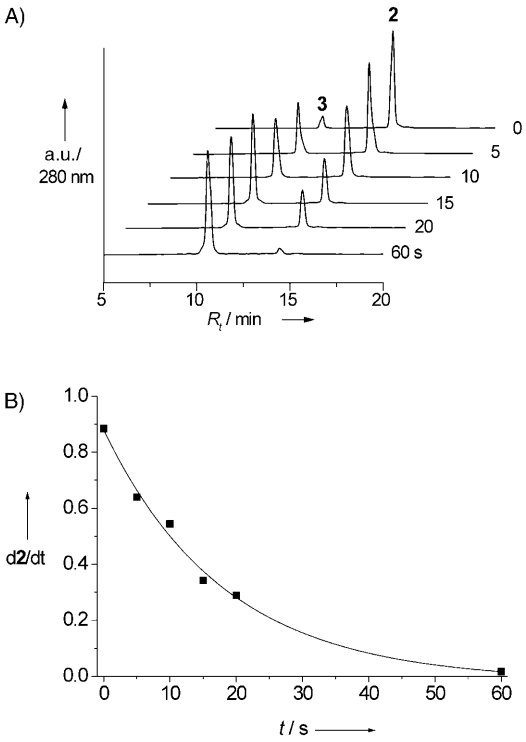

Figure 1. Time course of the photolytic-cleavage reaction of oligomer **2**. A) HPLC traces (280 nm) after UV irradiation for the indicated time. B) Normalized fraction of residual starting material **2** fitted with a first order exponential decay.

this unit in the center of the sequence. RNA synthesis was performed on a commercial DNA synthesizer by using standard protocols. 2′-*O*-*t*-Butyldimethylsiloxy (TBDMS) phosphoramidites were used as regular RNA building blocks. The coupling time was set to 12 min for unit **1**, and 5-(ethylthio)-1*H*-tetrazole was used as activator. According to the standard trityl assay, all steps in the synthesis proceeded with coupling yields of $>98\%$. Detachment from solid support was followed by base and phosphate deprotection with concentrated NH_3/EtOH (4:1) at 55 °C for 16 h. Subsequently, the silyl groups were removed with 1 M tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (THF). RP-HPLC purification afforded the abasic RNA 7-mer precursor **2**, the mass of which was confirmed by ESI-MS (see Supporting Information).

We then determined the kinetics of cleavage of the photolabile 1NPE group. An aqueous solution of **2** was irradiated with a UV lamp (see Experimental Section), and the amount of 1NPE cleavage as a function of time was quantified by HPLC (Figure 1). The half-life for 1NPE photolysis was determined to be 13 s. Deprotection was virtually quantitative after 1 min, as can be seen from the HPLC traces and as was found by ESI-MS data ($>99.8\%$). Interestingly, besides the molecular mass at *m/z* 2085.9, the MS of the deprotected abasic RNA **3** showed an additional peak with a mass difference of $\Delta m/z = +18$. This indicates that the aldehyde function at the abasic site might exist to a non-negligible extent in its hydrated form in solution. A thorough analysis of the chemical stability and kinetics of decay of abasic RNA in comparison to DNA is currently underway and will be published in due course.

RNA can also act as genetic material as, for example, is the case for retroviruses. In order to determine the potential mutagenicity of an RNA abasic site we studied its effect on reverse transcriptase. This enzyme is responsible for reverse transcription of a retroviral RNA single strand into a DNA single strand. Subsequent hydrolysis of the RNA in the hybrid duplex and synthesis of the complementary DNA strand result in a DNA duplex that contains the original RNA information. We chose HIV-1 reverse transcriptase as a model enzyme due to its well-characterized structural and biophysical properties.[10] We synthesized a 31-mer RNA template–DNA primer system that contained the abasic-RNA site adjacent to the 3′-end of the DNA primer (Figure 2). In order to follow translesion synthesis, the primer was ^{32}P labeled at the 5′ end by using T4-polynucleotide kinase. The purified, 1NPE-protected RNA template was annealed to its DNA primer and subjected to UV irradiation for 2 min to reveal the abasic site. The protected 1NPE-containing template–primer duplex was used as a control. Both binary mixtures were then incubated with each of the four dNTPs and enzyme at two different concentrations. The reactions were quenched after 60 min at 37 °C and the products separated by PAGE and visualized on a Phosphorimager (Figure 2).

Inspection of the gel revealed that HIV-1 reverse transcriptase readily bypassed RNA abasic sites at concentrations as low as 0.5 units and yielded full-length products in the presence of all four dNTPs (Figure 2 A). Elongation is not as efficient as in the case of an intact template strand (X = U). Experiments with single dNTPs show that dATP is preferentially inserted op-

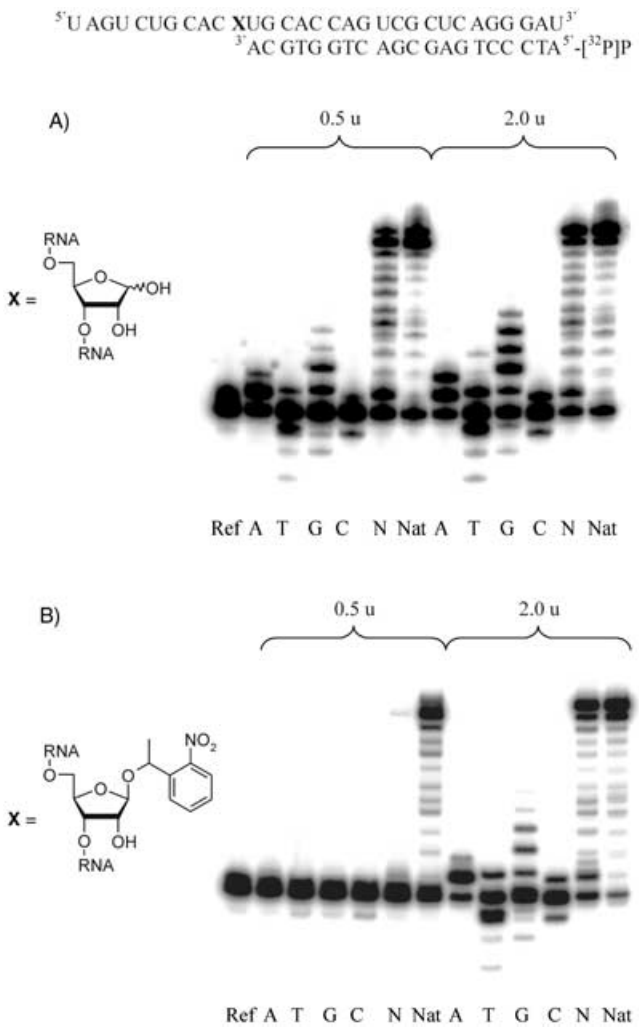

Figure 2. Denaturing PAGE gel (20%) of primer–template elongation products obtained with HIV-1 reverse transcriptase at 37 °C for 60 min. A) Abasic-site template; B) 1NPE-protected template; enzyme concentrations 0.5 and 2.0 units. Ref: without enzyme and dNTPs. A, T, G, C: reactions in presence of the corresponding dNTPs; N: reactions in presence of all four dNTPs; Nat: unmodified template (X = U) and all four dNTPs.

posite the abasic lesion. Qualitatively, the dNTPs are incorporated in the order A > G > T ≈ C. Some exonuclease activity is observed on the primer strand in the case of (slow) pyrimidine-nucleotide incorporation.

We also tested the template strand that contained the protected abasic residue (Figure 2B) for dNTP incorporation by HIV-1 reverse transcriptase. This experiment was performed so as to determine the difference between an abasic site and a noncoding, non-hydrogen bonding, stereochemically demanding base replacement. In contrast to the situation at the abasic site, essentially no dNTP incorporation was observed at low enzyme concentration. Incorporation and full-length extension were observed only at fourfold higher enzyme concentration. Again, dA was preferentially introduced opposite the bulky 1NPE group, while the other three dNTPs were incorporated at substantially slower rates.

We also checked for the RNase H activity of HIV-1 reverse transcriptase. For this, the abasic (X = AS) and unmodified (X = U) template strands were ^{32}P 5′-end labeled and the primer was not labeled (Figure 3).

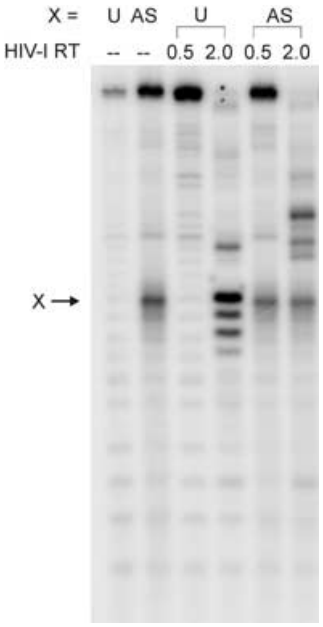

Figure 3. RNase H activity of HIV-1 reverse transcriptase on primer–template complexes that either contained the unmodified template (X = U) or the RNA abasic site (X = AS). Experimental conditions were the same as those used for primer-elongation experiments. HIV-1 reverse transcriptase (RT) concentrations are indicated.

Control experiments performed in the absence of HIV-1 reverse transcriptase (Figure 3, left 2 lanes) show that the RNA template is partially cleaved at the abasic site, presumably by β-elimination at the 3′ terminus of the RNA. We assume, however, that most of this cleavage occurs under denaturation conditions (5 min, 90 °C) prior to application to the gel and not during incubation. We note that partial cleavage is not expected to interfere with primer extension as the RNA template is used in excess. At low enzyme concentration essentially no RNase H activity is observed. Increasing the enzyme concentration to 2 units results in complete disappearance of the full-length RNA template. Interestingly, there are differences in the RNA degradation pattern as a function of the presence or absence of the RNA abasic site. In the unmodified RNA template, cleavage occurs predominantly at the junction between the double-helical part and the RNA single strand. However, in the abasic template, cleavage is effected predominantly in the double helical primer–template region. This differential cleavage pattern is in accord with the enzyme predominantly cleaving in the unextended primer part due to slow elongation in the latter case.

We draw the following conclusions from these preliminary results: phosphoramidite **1** is a useful building block for the synthesis of abasic RNA. It is readily incorporated by conventional RNA solid-phase synthesis, and the 1NPE group is almost quantitatively cleaved after 1 min of irradiation with a UV immersion lamp. Furthermore, an RNA abasic site is readily by-

passed by HIV-1 reverse transcriptase. As in the case of DNA templates,[11] a deoxyadenosine residue is preferentially introduced opposite the abasic site; this demonstrates that the "A rule"[12] is also valid when the template is an abasic RNA strand. In addition, RNase H activity is not inhibited by the presence of an abasic site. Work towards a comprehensive kinetic characterization of reverse transcriptase action on abasic RNA templates is currently underway.

The study of the biological role of abasic RNA has so far been hampered by the lack of a reliable method for its synthesis. With this technique at hand it might now become possible to incorporate such lesions into larger, biologically relevant RNA molecules by, for example, splint ligation methods.[13] The study of such constructs will be of interest, for instance, in the context of RNA viral evolution, rRNA function, mRNA translation, and of a possible existence of an RNA repair mechanism.

Experimental Section

Synthesis, deprotection, and purification of oligonucleotides: All oligonucleotides were prepared by automated oligonucleotide synthesis with an Expedite 8900 nucleic-acid synthesis system (PerSeptive Biosystems, Inc., Framingham, MA) by using the cyanoethylphosphoramidite approach. For RNA synthesis 2′-*O*-TBDMS protected PAC-phosphoramidites (GlenResearch) and polystyrene solid supports (Amersham Biotech) were used. For DNA synthesis benzoyl (dA, dC) and isobutyryl (dG) protected phosphoramidites and controlled pore-glass solid supports (GlenResearch) were used. The synthesis was performed by using the same standard coupling protocol for DNA and RNA with 5-(ethylthio)-1*H*-tetrazole (Aldrich) as activator and a coupling time of 90 s for DNA and 6 min for RNA. The modified phosphoramidite was allowed to couple for 12 min. Solid supports were treated with EtOH/NH_4OH (1:4) at 55 °C, overnight. RNA sequences were further deprotected by treatment with Bu_4NF (1 M in THF) for 6 h at RT. Oligonucleotides were desalted by using Sep-Pak® C_{18} columns (Waters, Milford, MA) before application to HPLC.

The DNA primer for the primer–template extension reactions and the RNA heptamer were purified by using RP-HPLC on an ÄKTA 900 HPLC system (Amersham Pharmacia Biotech). A Brownlee Aquapore RP-300 (C_8, 7 μm, 250/4.6 mm) column (PerkinElmer) was used with a gradient of solvent A (0.1 M Et_3NOAc in H_2O, pH 7.0) and solvent B (0.1 M Et_3NOAc, CH_3CN/H_2O (4:1)). The 31-mer RNA templates were purified on a 20% preparative denaturing (7 M urea) polyacrylamide gel. The oligoribonucleotides were then electroeluted with an Elutrap® electroelution system (Schleicher & Schuell) according to the manufacturer's protocol and desalted by using Sep-Pak® C_{18} columns. Purified oligonucleotides were dissolved in DEPC-treated water and the concentration was determined by using a NanoDrop® ND-100 UV/Vis spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE).

Kinetics of 1NPE deprotection: 1NPE-protected oligonucleotides were deprotected at RT by using a UV immersion lamp TQ 150 (UV-RS-2, Heraeus). Aliquots (0.4 mL) of a 1 $OD_{260}\,mL^{-1}$ solution of 1NPE-protected RNA heptamer **2** were exposed to UV light for different time intervals in a quartz cuvette and immediately analyzed by RP-HPLC. The ratio of **2**:**3** was determined by peak integration. From the first order rate law for disappearance of **2** we calculated a deprotection extent of >99.99% after irradiation for 2 min. Integration of ESI-MS peaks at the *m/z* of **2** and **3** confirmed this result and showed a deprotection extent of >99% after 2 min.

HIV-1 reverse transcriptase assays: HIV-1 reverse transcriptase (Worthington Biochemical Corporation, Lakewood, NJ) was diluted in storage buffer (10 mM K_3PO_4, pH 7.4, 1 mM DTT, 20% glycerol). For the primer-extension experiments the DNA primer (30 pmol) was labeled by using T4-polynucleotide kinase (10 units, Fermentas) and [γ-^{32}P]ATP (Hartmann Analytik GmbH, Braunschweig, Germany) in T4 buffer (50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA) for 30 min at 37 °C. T4-Polynucleotide kinase was then inactivated by heating to 90 °C for 2 min. For the RNase H assay the RNA templates (X = U or 1NPE) were 5′-end labeled as described above and used together with unlabeled DNA primer. The 1NPE-protected RNA template and DNA primer were annealed in a molar ratio of 2:1 in HIV-1 reverse transcriptase buffer. For the assays with abasic RNA this mixture was irradiated with a UV lamp for 2 min as described before. Final reaction mixtures contained RNA template (100 nM), DNA primer (50 nM), and dNTP (20 μM) in buffer (50 mM Tris pH 8.3, 50 mM NaCl, 8 mM $MgCl_2$, 1 mM DTT). After addition of the enzyme the mixtures were incubated for 60 min at 37 °C. The reactions were then quenched with loading buffer (98% formamide, 0.1% xylene cyanol (FF), 0.1% bromophenol blue), heated to 90 °C for 5 min and applied to a denaturing PAGE gel (20%). Radioactivity was detected and quantified on a Storm 820 Phosphorimager with ImageQuant software (Amersham Biosciences).

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