Base Pairing and Miscoding Properties of 1,N^6-Ethenoadenine and 3,N^4-Ethenocytosine Containing RNA Oligonucleotides

Alessandro Calabretta and Christian J. Leumann*

Department of Chemistry and Biochemistry, University of Bern, Freiestrasse 3, CH-3012 Bern, Switzerland

* Corresponding author. Tel:+41 31 631 4355; Fax:+41 31 631 3422; Email: leumann@ioc.unibe.ch:

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ABSTRACT

Two RNA phosphoramidites containing the bases 1,N^6-ethenoadenine and 3,N^4-ethenocytosine (εA and εC) were synthesized. These building blocks were incorporated into two 12-mer oligoribonucleotides for evaluation of the base pairing properties of these base lesions by UV-melting curve (T_m)- and circular dichroism (CD) measurements. The T_m data of the resulting duplexes with the etheno modifications opposing all natural bases showed a substantial destabilization compared to the corresponding natural duplexes, confirming their inability to form base-pairs. The coding properties of these lesions were further investigated by introducing them into 31-mer oligonucleotides and assessing their ability to serve as templates in primer extension reactions (PEX) with HIV-,AMV- and MMLV-reverse transcriptases (RT). Primer extension reactions showed complete arrest of the incorporation process using MMLV- and AMV-RT, while HIV-RT preferentially incorporates dAMP opposite to εA and dAMP as well as dTMP opposite εC. The properties of these RNA lesions are discussed in the context of its putative biological role.

The accumulation of DNA nucleobase lesions has been correlated with the onset of various diseases, first and foremost to most types of cancer.(*1-4*) The formation of such lesions has been extensively investigated in the past and can be attributed to a large variety of endogenous or exogenous agents.(*1*) In particular, oxidative stress is considered as one of the most important processes leading to damaged nucleobases, yielding different classes of chemical modifications that can accumulate on the same target sequence.(*4*) Reactive oxygen species (ROS) are endogenous compounds such as hydrogen peroxide, superoxide and hydroxyl radicals. These ROS are constantly produced during the normal mitochondrial aerobic respiration, during inflammation induced by viral or bacterial infections, by cytochromes P-450, and by the peroxisome-mediated degradation of fatty acids (*5*). Furthermore, ROS can attack nucleic acids in a direct or indirect way: in the first case they react directly with the nucleobases, while in the second case, they react first with cellular metabolites, forming intermediate species that introduce other types of lesions into the nucleobases.(*5*) 8-oxo-dA, 8-oxo-dG, 5-hydroxy-dU and 5-hydroxy-dC (*6*) are the main lesions that are produced via the first pathway while cyclic adducts such as etheno-dA, etheno-dC and etheno-dG (Figure 1) are mainly produced during the indirect path.

Etheno(ε)-adducts were first identified as products of the reaction of chloroacetaldehyde with natural nucleobases, nucleosides, and with DNA and RNA strands (*8-12*). In living organisms chloroacetaldehyde occurs as a metabolite produced from vinyl chloride as a xenobiotic (*13*). More recently, ε-bases have been shown to occur during lipid peroxidation (LPO) by the interaction of the resulting reactive aldehydes and hydroxyalkenals with DNA (*14-16*). Indeed, high concentrations of ε-bases have been identified in those cells where high amounts of

oxygen reactive species are produced indirectly, such as white blood cells during chronic inflammation.(*2*) The mutagenicity of these lesions when introduced into oligodeoxynucleotides has been fully investigated by *in vitro* and *in vivo* assays. and shown to be strongly mutagenic when tested in human kidney cell lines.*(17)* In this case, A/G transition and A/T transversion mutations were predominant.(*18, 19*) In other cells, such as *Escherichia coli*, minor mutagenic properties were observed for this lesion.(*19, 20*). Similarly, when tested *in vivo*, εC showed higher mutagenic properties with mammalian cells than with *E. coli*. Indeed, while mainly C/T transitions were observed in mammalian cells, only C/A transversions were produced in *E. coli*, albeit with a lower frequency.(*20-23*)

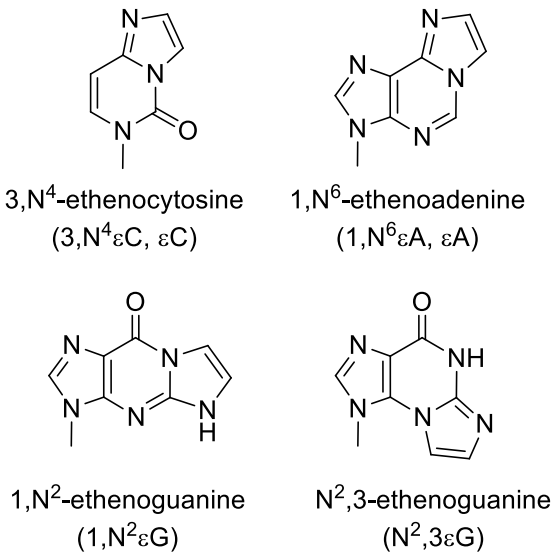

Figure 1. Chemical structures of the main etheno-base lesions

Although the formation of DNA base damage and its biological consequences is nowadays fairly well understood, surprisingly little is known on the effect of base lesions in RNA biochemistry and biology. Indeed, RNAs play a major role not only in translation (mRNAs, tRNA) but also in cellular regulation (miRNAs and other non-coding RNAs). Moreover, RNA is more abundant than DNA, is located in the

cytoplasm where most of the reactive species (in particular ROS) are present, and is less protected by proteins compared to histone organized DNA. Base damaged ribonucleosides such as 8-oxo-rA, 8-oxo-rG and 5-HOrU and 5-HOrC have recently been incorporated into oligoribonucleotides by solid-phase synthesis and their base pairing properties with complementary RNA as well as their coding preferences in templated primer extension reactions (PEX) by reverse transcriptases (RTs) have been assessed.(*24-27*) At the level of translation, oxidatively damaged mRNA has been demonstrated to cause largely diminished translation efficiency and abnormal protein synthesis, both *in vitro* and *in vivo*.(*28, 29*)

Here, we report on the synthesis of short 12-mer oligoribonucleotides containing either single εA or εC nucleotides as well as their base recognition properties upon duplex formation with complementary DNA and RNA. Furthermore, 31-mer RNA templates modified with these units were synthesized and used in PEX with three different RTs to determine the coding properties of these lesions during reverse transcription.

MATERIALS AND METHODS

Oligonucleotide synthesis and purification. Unmodified DNA and RNA oligonucleotides were purchased from Microsynth (Balgach, Switzerland) and HPLC or PAGE-purified when necessary. Natural 2'-TBDMS protected RNA phosphoramidites and CPG solid supports were obtained from GlenResearch or AZCO. The syntheses were performed on a 1.3 µmole scale on a Pharmacia Gene Assembler Plus DNA synthesizer following standard phosphoramidite protocols with

5-(ethylthio)-1H-tetrazole (0.25M in CH_3CN) as the activator and coupling times of 12 min. Oligoribonucleotides were deprotected using 33% NH_3/EtOH (3:1) for 16 h at 25°C in a screw cap 1.5 ml tube. Solid supports were filtered off and washed with NH_3/EtOH (3:1). The solutions were evaporated to dryness at room temperature in a Speedvac evaporator. The resulting pellets were further dried by addition of dry ethanol followed by evaporation. The pellets were then dissolved in anhydrous DMSO (100 µl) and neat triethylamine trihydrofluoride (125 µl) was added. After shaking at 25°C for 24 h, an aqueous solution of NaOAc (3 M, pH= 5.2, 25 µl) and n-butanol (1 ml) was added and the mixture was chilled on dry ice for 45 min. After centrifugation for 20 min in an Eppendorf, the liquid was discarded and the pellet washed twice with chilled ethanol (80%, 1 ml) and dried under high vacuum at room temperature, yielding the crude oligonucleotides. The 31-mer oligoribonucleotides were purified on a preparative 20% denaturing polyacrylamide gel (PAGE), the product band electroeluted with an Elutrap electroelution system (Schleicher & Schuell), and desalted using Amicon ultra 0.5 filters (MWCO 3KDa, Millipore). The 12-mer was HPLC purified on a DNAPac-200 column (4*250 mm, Dionex) with the gradient from 100% solvent A (25mM Tris, pH 8.0) to 50 % solvent B (25mM Tris, 1.25M NaCl, pH 8.0) in 30 min at room temperature, and finally desalted on a Sep-Pak C18 column (Waters Corp.). Oligonucleotide concentrations were determined using a NanoDrop ND-100 UV/Vis spectrophotometer (NanoDrop Technologies, Inc.). Stock solutions were made from DEPC treated H_2O.

UV-melting curves: Solutions were prepared in standard saline buffer (10mM NaH_2PO_4, 150mM NaCl, pH 7.0) with duplex concentrations of 2 µM in a 1:1 strand ratio. Thermal melting experiments were carried out on a Varian Cary 100-Bio UV/VIS spectrophotometer (Varian Inc.), equipped with a Peltier element at 260nm

with a heating/cooling rate of 0.5° C/min. T_m values were obtained from the maxima of the first derivatives of the melting curves using the WinUV software.

Labeling of oligonucleotides. The DNA primer d(ATCCCTGAGCGACTGGTGCA) was 5'-phosphorylated with [γ-^{32}P]ATP (Hartmann Analytic) and T4 polynucleotide kinase (Fermentas) as follows: DNA primer (30 pmol), [γ-^{32}P]ATP (60 mCi) and T4 PNK (10 U) were incubated in a total volume of 20 µl at 37°C for 30 min in T4 PNK buffer (50mM Tris–HCl, pH 7.6, 10mM $MgCl_2$, 5mM DTT, 0.1mM spermidine, 0.1mM EDTA). The T4 PNK was inactivated by heating the sample to 80°C for 2 min. The labeled primer was purified from unreacted free label by Amicon Ultra centrifugal filters, dried under vacuum and re-dissolved in 20 µl of DEPC water. Labeled DNA was then annealed to the RNA template (60 pmol) in the reaction buffer of the respective enzyme (see the description of RT assays) by heating to 60°C for 5 min and slow cooling down to room temperature.

RT assays. RT assays were performed with HIV-1 RT (Worthington Biochemical Corp), AMV RT (Promega Corp.) and MMLV RT (USB Corp./Affymetrix Inc.,) in the following buffers: HIV-1 RT and AMV RT: 50mM Tris (pH 8.3), 50mM NaCl, 8mM $MgCl_2$, 1mM DTT; MMLV RT: 50mM Tris (pH 8.3), 75mM KCl, 3mM $MgCl_2$, 10mM DTT. Final reaction mixtures contained RNA template (100 nM), DNA primer (50 nM) and dNTP (20 mM). After addition of the enzyme (2 - 40 U), the mixtures were incubated at 37°C for 1 h. The reactions were quenched with stop solution (98% formamide, 0.05% xylene cyanol (FF), 0.05% bromophenol blue), heated to 90°C for 5 min and resolved by 20% PAGE. Visualization and quantification were carried out on a Storm 820 phosphorimager with ImageQuant software (GE Healthcare).

RESULTS

Synthesis of the modified phosphoramidites and oligoribonucleotides. The RNA oligonucleotides containing εA and εC modifications were synthesized using the RNA phosphoramidites **1** and **2** depicted in Figure 2. The synthesis of these building blocks was performed via a modification of a literature procedure.(*30*) The experimental details and the analytical data of the synthesized compounds can be found in the Supporting Information.

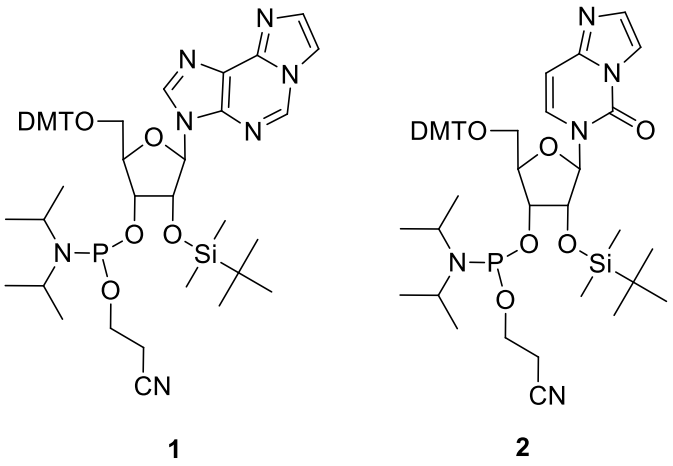

Figure 2. Chemical structures of the εA- and εC RNA-phosphoramidites **1** and **2**.

The oligoribonucleotides prepared for this study are summarized in Table 1. The 12-mer oligonucleotides ON 1-4, contain one of the damaged nucleobases or the corresponding natural bases in the center of the sequence and were used for T_m and CD-studies, while the 31-mer oligonucleotides ON 5-8 were used as templates in PEX.

Table1. Natural and modified sequences used in this work

Oligonucleotide	**RNA Sequence**
ON 1	5‘-AUGCU**εA**AGUCGA-3‘
ON 2	5‘-AUGCU**εC**AGUCGA-3‘
ON 3	5‘-AUGCU**A**AGUCGA-3‘
ON 4	5‘-AUGCU**C**AGUCGA-3‘
ON 5	5’-UAGUCUGCAC**εA**UGCACCAGUCGCUCAGGGAU-3’
ON 6	5’-UAGUCUGCAC**εC**UGCACCAGUCGCUCAGGGAU-3’
ON 7	5’-UAGUCUGCAC**A**UGCACCAGUCGCUCAGGGAU-3’
ON 8	5’-UAGUCUGCAC**C**UGCACCAGUCGCUCAGGGAU-3’

Coupling yields of the modified building blocks **1** and **2** were >98% as determined by trityl assay. Oligoribonucleotides were first base-, phosphate and finally 2’-*O* deprotected. The 12-mer sequences were purified by IE-HPLC, while the 31-mer oligonucleotides were PAGE purified. In order to limit the extent of ethenobase degradation in alkaline media,(*31*) we carried out the cleavage and base-deprotection in 33%NH_3:Ethanol =3:1 at 25°C overnight, obtaining the desired products in acceptable isolated yields: 14.8% for oligonucleotide ON 1 and 10.5% for oligonucleotide ON 2. HPLC traces of crude oligonucleotides (Figure 3) and mass spectrometric data (see Supporting Information) demonstrated the relative stabilities of the modified oligonucleotides during deprotection.

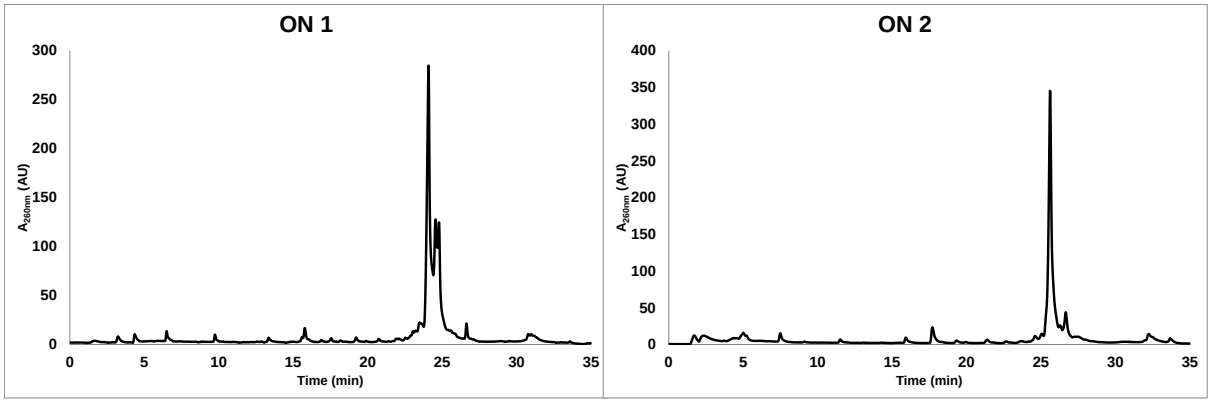

Figure 3. HPLC of crude ON 1 (left) and ON 2 (right)

UV-melting curves and CD spectroscopy. In order to study the influence of ε-lesions on the hybridization properties of RNA, melting curves of the duplexes containing the modified oligonucleotides ON 1 or ON 2 and the corresponding RNA or DNA complements were measured and compared with the corresponding natural duplexes containing oligoribonucleotides ON 3 and ON 4 (Table 2, Figure 4).

Table2. T_m data (°C, 260 nm) of duplexes of the oligoribonucleotides ON 1-ON 4 with r(UCGACUXAGCAU) or d(TCGACTXAGCAT) as complement; duplex concentration: 2 μM in 10mM NaH_2PO_4, 150mM NaCl, pH 7.0

	RNA Complements				**DNA Complements**			
X=	**rA**	**rC**	**rG**	**rU**	**dA**	**dC**	**dG**	**dT**
ON 1 (εA)	43.9	40.6	44.9	42.0	28.6	23.6	29.3	25.4
ON 3 (A)	44.9	46.6	46.7	57.6	29.6	34.6	29.9	45.3
ON 2 (εC)	43.9	39.3	46.6	41.9	25.2	20.8	25.3	24.3
ON 4 (C)	46.9	43.0	64.6	43.9	27.2	24.5	48.6	27.2

The T_m analysis further underscores the drastic influence that the exocyclic lesions have on the hybridization properties of nucleic acids. Indeed, the loss of their ability to form standard Watson-Crick hydrogen bonds (*32, 33*) causes a major destabilization of the RNA-RNA duplexes with the cognate complementary base opposite to the lesion (-15.6°C in the case of εA and -18.0°C in the case of εC, Table 2) . The same trend can be observed in the case of RNA-DNA duplexes. Moreover, the *T*m values are not further reduced when all the other nucleobases face the lesion, clearly showing that the natural base-lesion pair constitutes a mismatch, regardless of the nature of the base. In all cases, the duplexes formed with a G opposite the lesion are slightly more stable than the others, most likely due to stronger base stacking interactions.

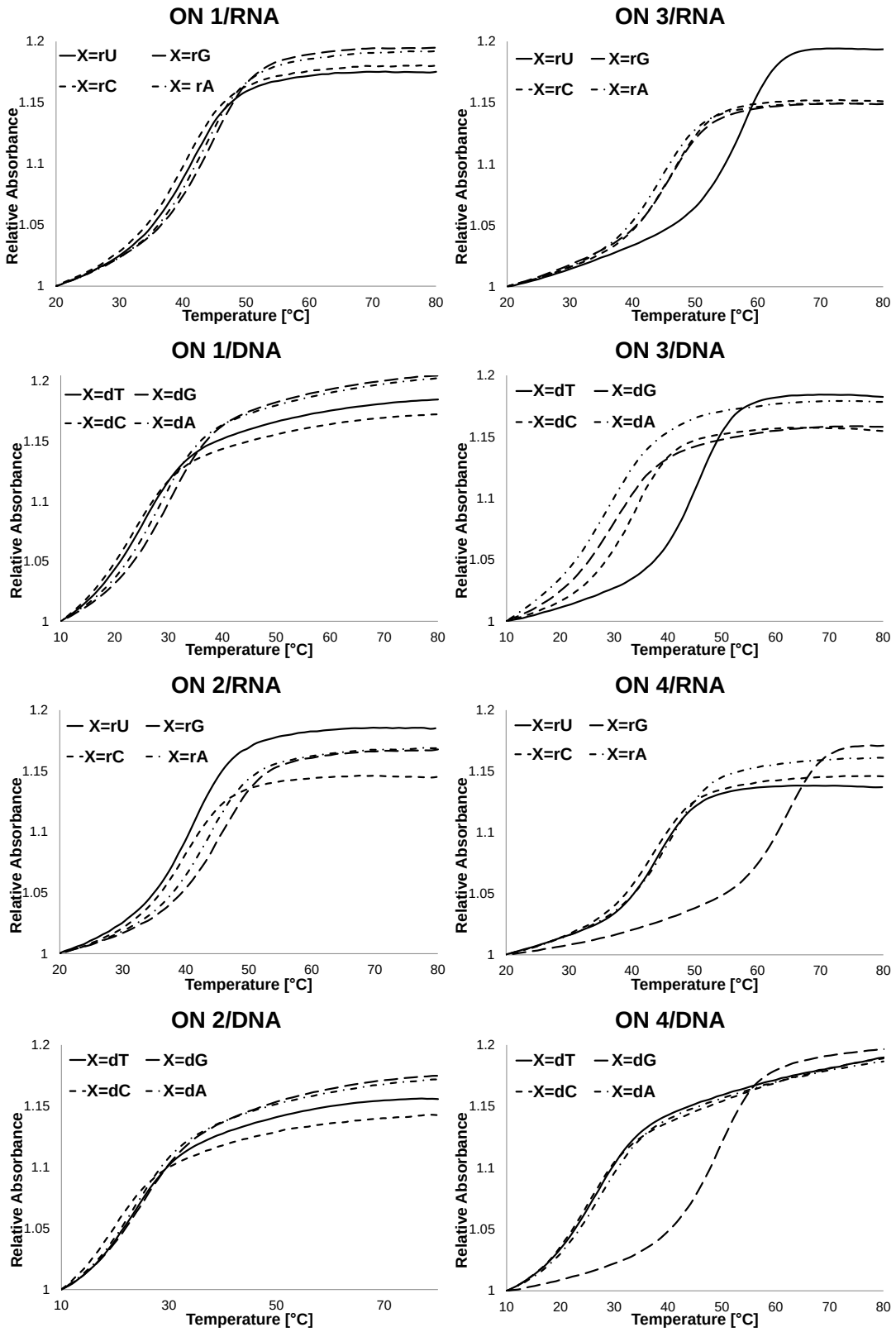

Figure 4. T_m curves for duplexes of oligonucleotides containing a lesion (ON1 and ON2) or the corresponding natural base (ON 3 and ON 4) with r(UCGACUXAGCAU)

or d(TCGACTXAGCAT); duplex conc.: 2 µM in 10mM NaH_2PO_4, 150mM NaCl, pH 7.0.

To study the influence of these exocyclic base lesions on the structure of the duplexes, CD measurements were performed. ON 1 and ON 2 were hybridized with complementary RNA sequences and the CD-spectra of the resulting duplexes all showed a standard A-conformation (Figure 5) and do not deviate substantially from the corresponding unmodified duplexes (data not shown).

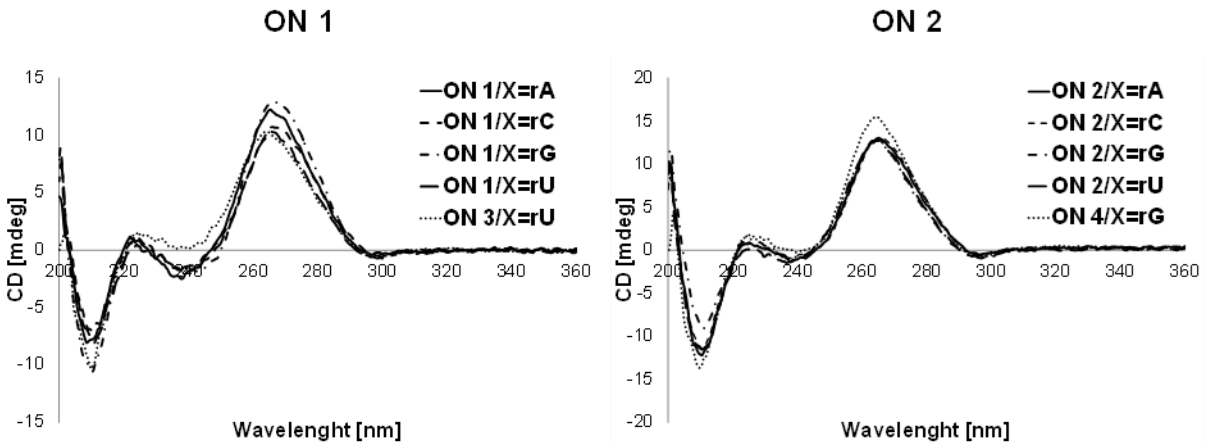

Figure 5. CD spectra of duplexes containing ON 1 or ON 2 with r(UCGACUXAGCAU). Duplex concentration: 2 µM (1:1 strand ratio) in 10mM NaH_2PO_4, 150mM NaCl, pH 7.0; T=25°C.

Primer template extension experiments. Further investigations of the coding properties of the ε-bases were performed using standing-start template primer extension reactions in a similar setup as reported previously for other RNA lesions.(*27, 34*) 5'-^{32}P radiolabeled DNA primer, dNTPs, 31-mer templates containing the lesions and three different RTs having different transcription fidelities

(HIV1-RT, AMV-RT and MMLV-RT) were used and the corresponding gels are shown in Figures 6 and 7. Quantitative data of dNMP incorporation opposite the lesion is given in the Supporting Information.

When the transcriptases AMV-RT and MMLV-RT were used, complete inhibition of reverse transcription was observed for both the εA and εC lesions. Even in the presence of all the natural dNTPs, no bands or only minor traces corresponding to elongated primer could be observed (Figures 6 and 7). On the other hand, a different behavior was observed in the case of HIV-RT. This error prone polymerase is able to read through both damages. In the case of the εA containing RNA template (ON 5), dAMP (78.3 %) was the most efficiently incorporated nucleotide, followed by dGMP (71.3 %). No incorporation of the base thymine (dTMP) could be observed (Figure 6). Thus, the order of preferential incorporation opposite εA is A>G>>C,T. In the presence of all dNTPs, full length synthesis was observed although being slightly less efficient compared to the undamaged template.

The results obtained using the εC containing RNA template (ON 6) are shown in Figure 7. In contrast with what was observed in the case of the εA containing template, HIV1 RT was completely unable to incorporate dGMP opposite εC. As observed for ON 5, the most efficiently incorporated nucleotide was dAMP (79.2 %). Moreover, the incorporation of dTMP was rather efficient (61.8 %) but not as good as that for dAMP. The analysis of these results lead to the following preferential order of incorporation opposite εC: A>T>>C>G. Again, in the presence of all the dNTPs, synthesis of the full-length product is observed and the lesion seems to have no inhibitory effect.

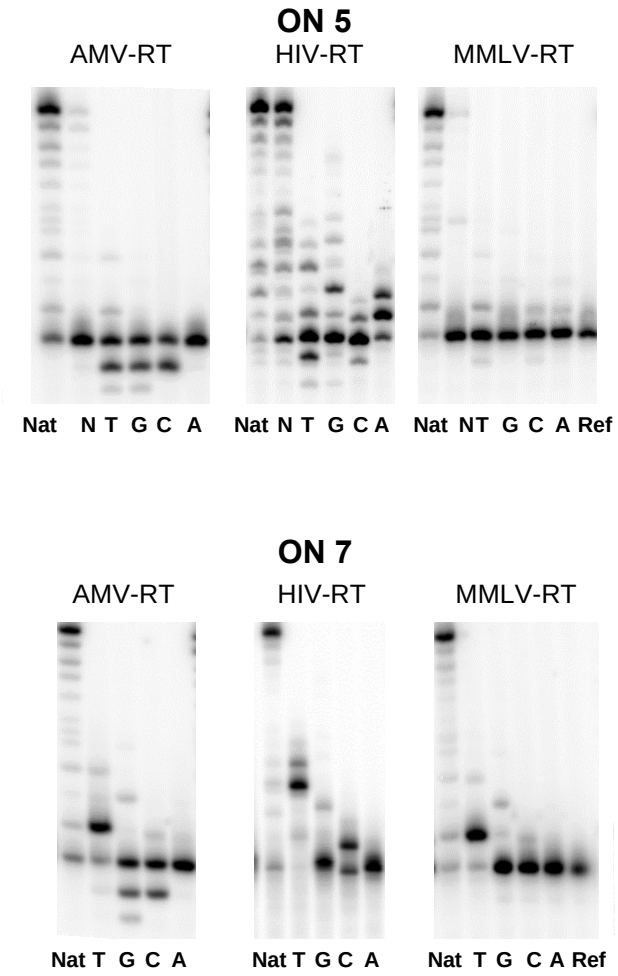

Figure 6. Autoradiograms of standing-start primer extension reactions for the RTs indicated using ON 5 containing the εA lesion as the template and the corresponding natural template (ON 7). Reactions were performed at 37°C for 1 h. Enzyme concentrations: HIV-RT, 2 U; AMV-RT, 8 U; MMLV-RT, 40 U. Ref: primer without enzyme; A, T, G, C: reactions in presence of the respective dNTP; N: reactions in presence of all four dNTPs; Nat: unmodified RNA template and all four dNTPs.

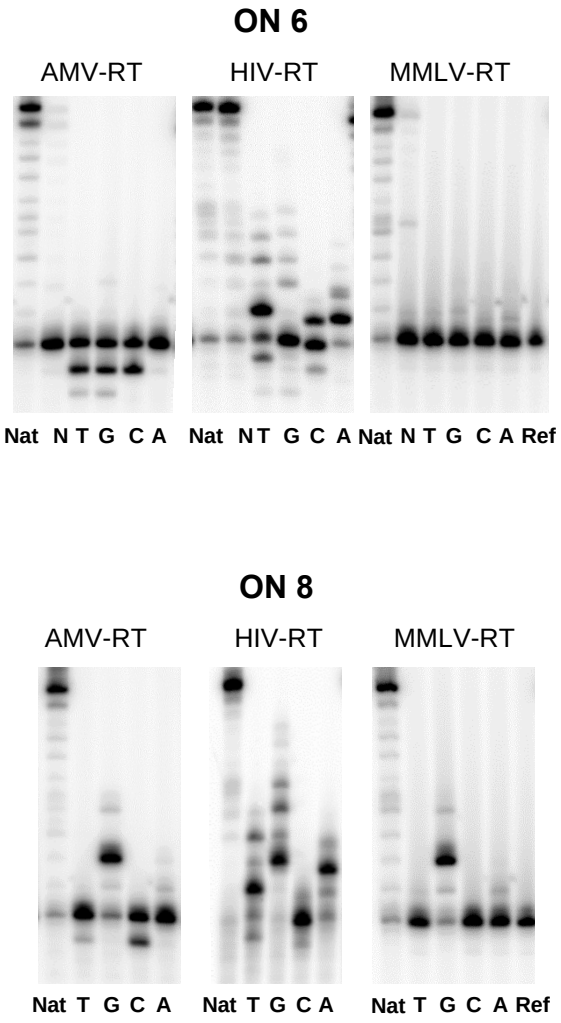

Figure 7. Autoradiograms of standing-start primer extension reactions for the RTs indicated using ON 6 containing the εC lesion as a template and the corresponding natural template (ON 8). Reactions were performed at 37°C for 1 h. Enzyme concentrations: HIV-RT, 2 U; AMV-RT, 8 U; MMLV-RT, 40 U. Ref: primer without enzyme; A, T, G, C: reactions in presence of the according dNTP; N: reactions in presence of all four dNTPs; Nat: unmodified RNA template and all four dNTPs.

DISCUSSION

We describe here the chemical synthesis of oligoribonucleotides containing 1,N^6-ethenoadenosine and 3,N^4-ethenocytidine using standard phosphoramidite chemistry. Although the synthesis of such RNA oligonucleotides was previously reported,(*30*) to the best of our knowledge the pairing and coding properties of oligoribonucleotides containing such lesions have never been reported. From UV-melting curve analysis it appears that both etheno modifications completely lack recognition capacities towards all natural bases. The slightly more stable arrangements with purine bases opposite the etheno lesions may be explained by more favorable stacking interactions. These results are in agreement with earlier findings on the hybridization properties of oligodeoxynucleotides containing εdA or εdC lesions.(*35-37*) Even there, strong duplex destabilization against any natural base was observed. Again, in these cases the most stable duplex resulted when a G was placed opposite to the lesion.(*35, 36*) confirming the importance of (presumably interstrand) stacking interactions on duplex stability.

The structure of the duplexes containing these lesions was investigated by CD spectroscopy. The results indicate that their presence do not affect the overall A-helix conformation of the duplexes. Thus, the expected local structural perturbations at the site of the base lesion are (not unexpectedly) invisible by CD-spectroscopy. Clearly more high resolution structural work by NMR or X-ray crystallography is needed to reveal the precise arrangement of the modified bases.

The primer extension experiments nicely show the mutagenic potential of εA and εC during reverse transcription. While none of the two etheno nucleosides investigated can be read by AMV or MMLV RT, leading to abortion of DNA synthesis,

both of them are accepted by the more permissive HIV RT. However, depending on the nature of the lesion, the coding preferences are somewhat different. Indeed, εA preferentially leads to purine incorporation opposite itself with a slight advantage for dAMP (A-rule). This behavior has been observed in a similar experiment with an abasic site as a non-coding lesion in an RNA template.(*34*)

Primer extension experiments using εdA inserted in a DNA template were reported previously.(*38*) Surprisingly, from these experiments it resulted that the only enzyme completely inhibited by the presence of this lesion is HIV-RT. Quite interestingly, MMLV-RT could elongate the DNA primer in different sequence contexts, although not to full length. This again is contrary to what we observed with the RNA template. It therefore seems that HIV RT shows a higher degree of sensitivity towards lesions on DNA compared to RNA. This may imply that retroviral mutations are more likely to occur during DNA synthesis on the viral RNA template than during complementary DNA strand synthesis on the reverse transcribed DNA strand.

Also in the case of etheno cytidine there is precedence in the literature on its coding properties when present in a DNA template. (*39*) It needs to be noted though that these PEX were performed with DNA polymerases and not with reverse transcriptases. Compared with our results with HIV RT, lesion bypass synthesis is more strongly slowed down for all of the DNA polymerases investigated in this study. The nature of the bases preferentially introduced opposite the lesion is highly dependent on the polymerase used. Indeed, Pol α incorporates dAMP and dTMP opposite to the lesion, pol β dAMP and dCMP, pol η dAMP and dGMP, and pol κ dTMP.(*39*) Thus, the preferences of HIV1 incorporation in our experiments compare

well to those of pol α. Again, we need to point out that these conclusions do not take into consideration variations in base preferences as a function of the sequence context.

The mutagenicity of base lesions in DNA is a major threat to genome integrity of living systems. In part this is also true for base damaged RNA in organisms, the genome of which consists of RNA (e.g. RNA viruses). Moreover, base damage to RNA can have additional biological implications. For example, it can deeply affect the cellular metabolism if non-coding RNAs involved in regulatory processes are affected. In addition, if present on mRNAs, such base lesions can lead to misincorporations of amino acids during translation (translational mutations), or alternatively it can lead to ribosome arrest which may severely impair cellular vitality.

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SUPPORTING INFORMATION AVAILABLE

Synthetic and analytical details for phosphoramidites **1** and **2**, mass spectra for etheno modified oligonucleotides as well as quantitative data on dNMP incorporation for primer extension reactions. This material is available free of charge via the Internet at http://pubs.acs.org.

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