A primer extension assay for modification of guanine by Ni(II) complexes

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The selective modification of RNAs by chemical reagents is now widely used to study their solution structure (reviewed in Refs. 1 and 2). These methods have the advantage of requiring only small amounts of material and can be applied to large transcripts. The information obtained from chemical protection is complementary to comparative sequence analyses. Structural interpretation of data usually depends on selective modification of nucleotides that are accessible to solvent. Sites of base modification are either detected by strand scission in the presence of aniline acetate (3), or by extension of a primer with reverse transcriptase (4, 5). The former has the advantage of being direct and is convenient for small end-labeled RNAs. The latter approach, however, avoids aniline treatment and the need for an end-label, and consequently can be used with large RNA molecules and RNA-protein complexes.

To generate reliable structural information, it is advantageous to use a variety of reagents with differing specificities. To this end, we have developed a new inorganic probe for nucleic acid structure. In particular, guanine is selectively oxidized by a planar Ni(II) complex, NiCR, (Figure 1) in the presence of KHSO₅ (6). The extent to which G's in oligodeoxynucleotides and yeast tRNA^{phe} are modified by NiCR correlates well with the solvent accessibility of G N7 (7, 8). The utility of this reagent for RNA structure has been further demonstrated by its application to the *Tetrahymena* intron (8).

Previously, RNA modification by NiCR was detected by strand cleavage in the presence of aniline (8), making the reagent inconvenient to use with large molecules. While methylation of a purine N7 by dimethylsulfate cannot be detected by primer extension, we reasoned that a bulky substituent at that position might cause a pause in reverse transcription. A second nickel complex, Ni-TMAPES (Figure 1), has been recently shown to alkylate DNA under moderately oxidizing conditions (9). The selectivity of this reagent towards single-stranded or non-canonical base-paired guanosines in DNA oligomers is very similar to that of NiCR (9). Selectivity towards the N7 of guanosine distinguishes these reagents from kethoxal, which forms a covalent adduct with N1 and N2 and is commonly used to identify unpaired G residues. Thus it appeared that this reagent might be useful in chemical protection studies that employ primer extension. In this communication, we show that both alkylation of the *Tetrahymena* intron RNA by Ni-TMAPES and oxidation by NiCR are readily detected by reverse transcription. A method for probing RNA structure with Ni(II) complexes is described.

Reagents

NiCR, **1**, (2,12-dimethyl-3,7,11,17-tetraazabicyclo[11.3.1]heptadeca-1(17),2,11,13,15-pentaenato)-nickel(II) perchlorate, was prepared according to published procedures (10). Ni-TMAPES, **2**, [N,N'-bis(salicylaldehyde)-*meso*-1,2-bis(4-trimethylaminophenyl)-ethylenediimino]nickel(II) perchlorate, was synthesized as previously described (9). Solutions of magnesium monoperoxyphthalate (MMPP, Aldrich) were prepared in deionized water immediately before use.

RNA

L-21 Sca intron RNA was prepared as previously described (11). Prior to use in chemical protection experiments, the RNA (0.5 μ M final concentration) was suspended in 20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, heated 1 minute at 95°C, and cooled rapidly in the presence of 5 mM MgCl₂. For experiments without magnesium, the RNA was heated and cooled in 20 mM Tris-HCl, pH 7.5 plus 2.5 mM EDTA.

Modification by Ni(II) complexes

4 μ L of 0.5 μ M RNA was added to 71 μ L of 50 mM Na-cacodylate, pH 7.5, 200 mM NaCl, 5 mM MgCl₂. Reactions were initiated with the addition of 1.5 μ L of 0.5 mM MMPP (10 μ M final) and 3 μ L of a 150 μ M solution of Ni-TMAPES in water (6 μ M final), or 1.5 μ L of 5 mM MMPP (100 μ M final) and 3 μ L of 150 μ M NiCR (6 μ M final). Reactions were carried out at 37°C for 30 min. and quenched with 15 μ L stop solution (1.5 M Na-acetate, pH 7.0, 1 M β -mercaptoethanol, 0.1 M EDTA, 0.65 μ g/ml tRNA). The RNA was precipitated with three volumes of ethanol, resuspended in 100 μ L of 0.3 M Na-acetate and precipitated with ethanol a second time. The final pellet was washed with 70% ethanol and dried completely.

Primer extension

The intron RNA was analyzed by primer extension as described previously (12). Briefly, modified RNA (0.5–1 pmol) was annealed with 0.4 pmol of 5' [³²P]-labeled primer (Oligos, Etc) in 3 μ L of buffer (50 mM Tris-HCl, pH 8.3, 60 mM NaCl, 10 mM DTT). Samples were incubated at 65°C for 3 min. and then placed immediately on ice. To this was added 2 μ L of a mixture containing 1 U/ μ L AMV reverse transcriptase (Life Sciences, St Petersburg), 1.0 mM each deoxynucleotide triphosphates (Pharmacia), and 15 mM magnesium acetate in the

annealing buffer above. Extensions were carried out for 15 minutes at 48°C and stopped with the addition of 5 μ L formamide plus tracking dyes. A sequence ladder of unmodified RNA was obtained in the same manner by adding the desired dideoxynucleotide triphosphate (80 μ M) to the reaction. The products were separated on a 6% polyacrylamide sequencing gel containing 8 M urea.

As shown in Figure 2a, treatment of the *Tetrahymena* intron RNA with NiCR or Ni-TMAPES results in specific and highly reproducible stops by reverse transcriptase. These bands, which correspond to a pause before a G residue, are not seen when the RNA is incubated in buffer (–Ni, +MMPP) or in the presence of oxidant alone (–Ni, +MMPP). The reaction profiles of Ni-TMAPES and NiCR appear to be nearly identical. NiCR is thought to oxidize and degrade the guanine ring (6), and could result in termination of reverse transcription 3' of the lesion.

Guanosines that are modified in the presence of nickel are indicated relative to the secondary structure of the intron in Figure 2b. At least 1 mM magnesium ion is required to maintain the folded structure of the intron RNA (13). In the absence of magnesium, we observe that certain guanosines (for example, G215) become more reactive towards nickel complexes, whereas the reactivity of others, such as G303, is enhanced by magnesium. These results are consistent with those reported previously for reaction of the L-21 intron RNA with NiCR (8). Thus a similar subset of guanosines are found to be reactive towards oxidation or alkylation by these nickel complexes, whether the modification is detected by strand cleavage in the presence of aniline or by primer extension with reverse transcriptase.

Ni(II) macrocyclic complexes are a new and reliable chemical modification reagent with a unique specificity for the N7 of guanosine. The experimental protocol described here was designed to be similar to those typically used for modification of RNA by dimethylsulfate and diethylpyrocarbonate. Consequently, these nickel-dependent probes can be conveniently incorporated into existing strategies for RNA structure determination.

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Figure 1. Structure of Ni(II) complexes. 1, NiCR; 2, Ni-TMAPES.

Figure 2. Modification of *Tetrahymena* intron by Ni(II) complexes. **A.** The L-21 Sca RNA was incubated with nickel reagents and oxidant for 30 minutes at 37°C as described in Methods. Reactions were carried out in the presence (+Mg²⁺) or absence (–Mg²⁺) of 5 mM MgCl₂. Notations above the lanes indicate: –MMPP, no oxidant added to the reaction; –Ni(II), no nickel reagent; CR, 6 μ M NiCR and 100 μ M MMPP; T, 6 μ M Ni-TMAPES and 10 μ M MMPP. Sequencing lanes were generated by reverse transcription of unmodified RNA in the absence (–) or presence (T, G, C, A) of dideoxynucleotide triphosphates. Primer IP311–22 is complementary to the intron from position 311 to 332. **B.** Sites of reaction with Ni-TMAPES are indicated relative to the secondary structure of the L-21 Sca RNA (adapted from Ref. 14). Solid arrowheads, + and –MgCl₂; open circles, –MgCl₂ only; open arrowheads, +MgCl₂. Only the sites of modification between positions 57 and 315 have been determined.