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Guide RNA-directed uridine insertion RNA editing *in vitro*

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Guide RNAs (gRNAs) have been proposed to mediate uridine (U) addition/deletion editing of mitochondrial mRNAs in kinetoplastid protozoa. The Us are proposed to be derived either from UTP by two successive cleavage–ligations or transesterifications, or from the 3′ end of the gRNA by the same mechanisms. We have demonstrated gRNA-dependent U insertions into a specific editing site of a pre-edited mRNA which was incubated in a mitochondrial extract from *Leishmania tarentolae*. The predominant number of U insertions was determined by the number of guiding nucleotides in the added gRNA, and the formation of a gRNA–mRNA anchor duplex was necessary for activity. UTP and α–β bond hydrolysis of ATP were required, and the activity was inhibited above 50–100 mM KCl. A gRNA-independent insertion of up to ~13 Us occurred in the absence of the added cognate gRNA; the extent of this activity was affected by sequences upstream and downstream of the edited region. Heparin inhibited the gRNA-independent U insertion activity and had no effect on the gRNA-dependent activity. Blocking the 3′ OH of the gRNA had little effect on the gRNA-dependent U insertion activity. The data are consistent with a cleavage–ligation model in which the Us are derived directly from UTP.

Keywords: guide RNA/kinetoplast DNA/ND7/RNA editing/uridine insertion

Introduction

The uridine (U) addition/deletion type of RNA editing (Simpson and Shaw, 1989; Hajduk *et al.*, 1993; Simpson *et al.*, 1993; Stuart, 1993; Simpson and Thiemann, 1995; Simpson and Emeson, 1996) that occurs in the kinetoplast mitochondrion of the kinetoplastid protozoa (Simpson, 1972, 1986, 1987) has been proposed to be mediated by small guide RNA (gRNA) molecules which are complementary to mature edited mRNA and contain the sequence information for the inserted and deleted Us in terms of guiding A and G nucleotides which can base-pair with the inserted U residues (Blum *et al.*, 1990; Pollard *et al.*, 1990; Corell *et al.*, 1993). Guide RNAs have a 5′ 'anchor' sequence complementary to the mRNA just downstream of the region to be edited. The formation of the anchor duplex is thought to be required for the initial interaction of the cognate gRNA and the pre-edited mRNA (Blum *et al.*, 1990). The non-encoded 3′ oligo(U) tail of the gRNA has also been proposed to form a duplex with the G+A-rich pre-edited region and stabilize the initial interaction (Blum and Simpson, 1990). A single gRNA mediates the editing of a single block of mRNA sequence, and multiple overlapping gRNAs mediate the 3′ to 5′ editing of pan-edited domains containing multiple blocks of editing (Maslov and Simpson, 1992).

Several models for the mechanism of RNA editing have been proposed. The original enzyme cascade model (Blum *et al.*, 1990) invoked a cleavage at the first mismatched base in the mRNA adjacent to the gRNA–mRNA anchor duplex, 3′-terminal addition of U by the mitochondrial terminal uridylyl transferase (TUTase) (Bakalara *et al.*, 1989) and ligation of the two cleavage products by the mitochondrial RNA ligase (Bakalara *et al.*, 1989). In a modified enzyme cascade model (Sollner-Webb, 1991, 1992), U residues are transferred from the 3′ end of the gRNA by two successive cleavage–ligations. In the transesterification model, the U residues are either transferred from the 3′ end of the gRNA by two successive transesterifications, producing gRNA–mRNA chimeric molecules as intermediates (Blum *et al.*, 1991; Cech, 1991), or directly from UTP (Cech, 1991).

The gRNA hypothesis has been verified for U deletions by the establishment of an *in vitro* system with a *Trypanosoma brucei* mitochondrial extract, in which deletions in the first editing site of synthetic ATPase 6 pre-edited mRNA could be manipulated in a predictable manner by varying the number of guiding nucleotides present in the added cognate gRNA (Seiwert and Stuart, 1994). Putative intermediates in the editing process were observed directly by end labeling and gel analysis, and the evidence suggested that the observed chimeric molecules represented non-productive end products rather than intermediates in the U deletion reaction (Seiwert *et al.*, 1996).

An *in vitro* U addition activity has been reported using a mitochondrial extract from *Leishmania tarentolae* (Peris *et al.*, 1994; Frech *et al.*, 1995). The cytochrome b (CYb) and NADH dehydrogenase 7 (ND7) substrate mRNAs became labeled with [α-^{32}P]UTP and the localization of the labeled Us was determined by RNase H digestion with specific oligonucleotides. In addition to the expected 3′ U additions from the mitochondrial TUTase activity, approximately half of the labeled Us were inserted into the pre-edited domain. This incorporation occurred in the absence of added gRNA, and we have shown by a primer extension assay that this U insertion activity was independent also of endogenous gRNA (Connell *et al.*, 1996).

Here we present the first evidence for gRNA-directed

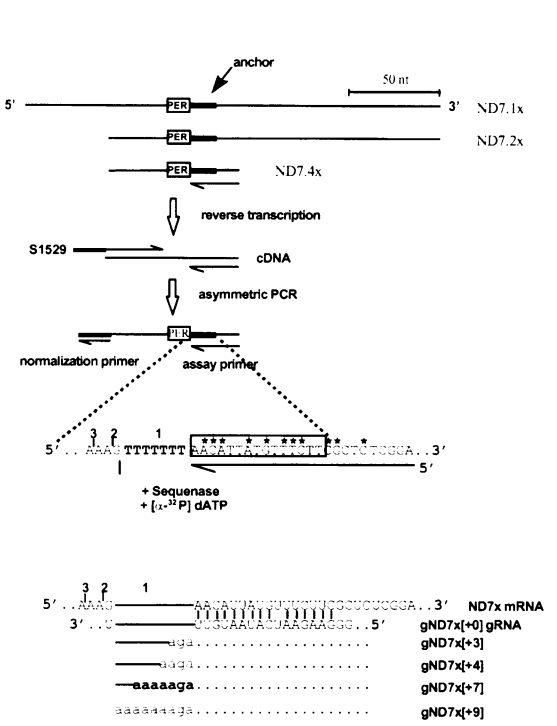

Fig. 1. Substrates and assay for detection of U insertions *in vitro*. (**A**) Diagram of indirect primer extension assay for U insertions in the first editing site of domain II of anchor-mutated (x) ND7 mRNA constructs. PER = pre-edited region. The mutated anchors in the three mRNA constructs are indicated by black boxes. At the bottom is the sequence of the first three editing sites in the mRNA (numbered), with the naturally occurring seven Us (Ts) shown in the first site. The anchor sequence is boxed and the mutated bases are indicated with asterisks. (**B**) Mutated anchor and first editing site of mRNA substrate shown aligned with complementary anchor and guiding nucleotides of gRNAs used for *in vitro* guiding experiments.

U insertions into a specific editing site of a pre-edited mRNA using a mitochondrial extract from a trypanosomatid protozoan. The results are interpreted in terms of the various models for the mechanism of U insertion/deletion RNA editing.

Results

The primer extension assay

The ND7 mRNA in *L.tarentolae* is edited in two domains (Shaw *et al.*, 1988, 1989). The 5′ domain has seven editing sites, and the mature edited mRNA has an insertion of seven Us in site 1, which is presumably mediated by base pairing with the seven guiding nucleotides (..agaaaaa..) in the cognate gRNA. In order to avoid detection of Us within endogenous edited ND7 mRNA and to prevent the endogenous ND7 gRNA from interacting with the synthetic substrate mRNA, the anchor sequence of the synthetic pre-edited substrate mRNA was mutated to yield the ND7x mRNA (Figure 1A), and compensatory mutations were made in the synthetic cognate gND7x gRNA (Figure 1B). Three different 3′-truncated ND7 mRNA constructs were used in these experiments: ND7.2x and ND7.4x have the 5′ end of the mature edited mRNA and possibly pre-edited mRNA (data not shown), while ND7.1x begins 45 nucleotides upstream; the ND7.4x construct is 100 nucleotides shorter than ND7.1x and ND7.2x at the 3′ end (Figure 1A). To avoid detection of Us derived from the oligo(U) tail of gRNAs covalently linked to mRNAs at site 1 in the form of gRNA–mRNA chimeras, the extension assay was performed on an amplified DNA copy of the ND7x substrate mRNA made by reverse transcription and asymmetric PCR, using a 3′ primer complementary to the mutated anchor sequence of the input mRNA and a 5′ primer specific to the mRNA sequence upstream of the pre-edited region. T (U) insertions in editing site 1 of the DNA were then detected by incorporation of [α-^{32}P]dATP into a primer extension product which terminated due to the absence of dCTP. A second primer annealing upstream adjacent to a T residue present in the 5′ PCR primer was included in order to normalize the gel loading (Figure 1A).

gRNA-dependent and gRNA-independent U insertion activities

The primer extension results in Figure 2A, several lanes of which are quantitated in Figure 2B, show that insertions of up to 13 or more Us were detected in site 1 of pre-edited ND7x mRNA after incubation with mitochondrial lysate. A primer +1 artifact band which was present without incubating the mRNA with the mitochondrial lysate was presumably due to a misincorporation during synthesis of the substrate and/or during the assay. This band interfered with detection of single U insertions but not with the detection of insertions of >1 U. In the absence of added gRNA, a ladder of labeled bands, consistent with the mRNA having been modified with up to ~13 U insertions, was observed with the ND7.1x substrate (lanes 13 and 15). The presence of a mutated anchor sequence in the mRNA indicates that this incorporation is also independent of endogenous gND7 gRNA. A weaker gRNA-independent U insertion activity was also seen for the ND7.2x mRNA (lane 2), while the ND7.4x mRNA showed little or no U insertion (lane 18).

Incubation of the anchor-mutated ND7x mRNAs with lysate in the presence of added cognate gND7x[+3] gRNA containing +3 guiding nucleotides at site 1 yielded U insertion ladders in which there were predominant labeled bands at the +3 position (lanes 9, 10, 16 and 19). The intensity of the +3 band was greater with the two shorter mRNA constructs (lanes 9 and 19). The addition of gND7wt[+3] gRNA, which has the wild-type anchor sequence together with three guiding nucleotides for site 1 editing, caused a decrease in the overall U insertion pattern for ND7.2x RNA (lane 4), compared with the signal generated without gRNA (lane 2) or with the cognate gND7x[+3] gRNA (lanes 9 and 10), and did not produce an enhancement of the +3 band. This indicates a requirement for an anchor duplex for gRNA-dependent U insertion activity. The addition of the heterologous gCYb gRNA to the reaction with the ND7.2x mRNA also produced an overall inhibition of U insertion without enhancement of any single band (lane 3).

Incubation of cognate gRNAs containing the naturally occurring +7 (gND7x[+7]), or +9 (gND7x[+9]) and +4 (gND7x[+4]) guiding nucleotides at site 1 with the ND7.2x mRNA substrate led to the appearance of prominent +7 and +9 and predominant +4 labeled bands, respectively. Use of gND7x[+0] gRNA, which contains no guiding nucleotides for site 1, with ND7.1x or ND7.2x mRNAs, produced a decrease in the background ladder

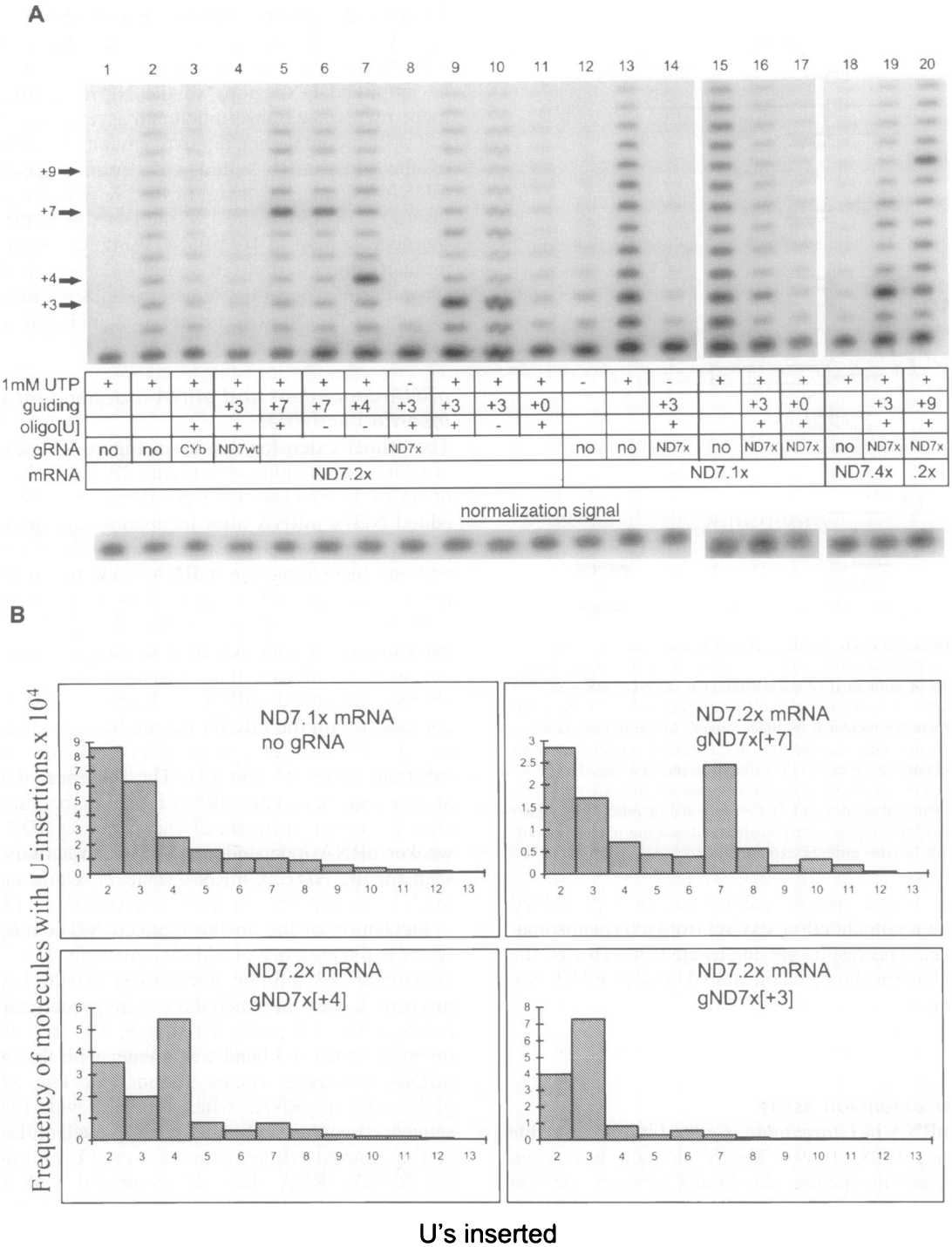

Fig. 2. *In vitro* insertion of Us in editing site 1 of synthetic ND7 mRNAs. (**A**) ND7x mRNAs incubated in mitochondrial extract in the presence or absence of 1 mM UTP, with or without gND7 gRNAs having the wild-type (wt) or the mutated (x) anchor. Guiding = the number of site 1 guiding nucleotides in the gRNA. Oligo(U) = the presence (+) or absence (–) of an oligo(U) tail generated during transcription of the gRNA. Lane 1, non-incubated control. The sizes of the extension products were determined by co-running a sequencing ladder (not shown). (**B**) Quantitation of lanes 13, 5, 7 and 9. Bands were quantitated by PhosphoImager analysis and the values normalized for loading and for the number of labeled deoxyadenosine residues by which the assay primer was extended. The fraction of substrate RNA with insertions of two or more Us in site 1 was calculated by comparison with the signal obtained from a synthetic ND7x mRNA with seven Us at site 1 mixed with pre-edited RNA in a 1/3000 molar ratio (data not shown).

below that observed in the absence of added gRNA, without any enhancement of a specific band. The use of ddCTP to terminate the primer extensions led to the appearance of predominant gRNA-guided extension products one nucleotide longer than those produced in the absence of ddCTP (data not shown), which is consistent with the presence of a guanosine residue 5′ of site 1 in the mRNA.

From the results in Figure 2A, which were quantitated in Figure 2B, it is clear that the absolute frequencies of

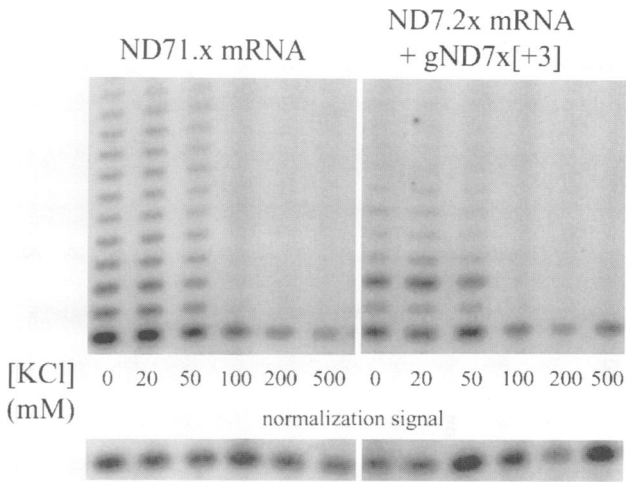

Fig. 3. Inhibition of gRNA-independent and gRNA-dependent U insertion activities by KCl. The extension ladders were obtained either using the ND7.1x mRNA without added gRNA or the ND7.2x mRNA with the gND7x[+3] gRNA. The reaction mixtures contained a range of concentrations of added KCl, and, as indicated in Materials and methods, standard incubation conditions included 20 mM added KCl.

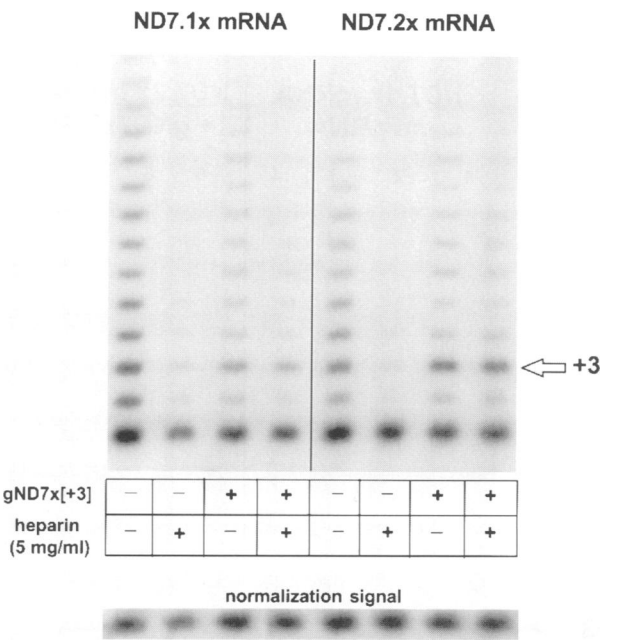

Fig. 4. Selective effect of heparin on gRNA-independent U insertion activity. On the left is shown the effect of heparin (5 μg/ml) on the gRNA-independent and gRNA-dependent U insertions obtained with the ND7.1x mRNA. On the right is shown the effect of heparin (5 μg/ml) on the gRNA-independent and gRNA-dependent U insertions obtained with the ND7.2x mRNA.

gRNA-dependent U insertions were quite low, in some cases approaching the frequency of the primer +1 artifact band. However, the presence of the primer +1 band was not dependent on incubation with lysate and did not interfere with visualization and analysis of the lysate-dependent and gRNA-dependent bands of interest.

Both the gRNA-dependent and the gRNA-independent U insertion activities required added UTP (Figure 2A, lanes 8, 9, 14 and 16; 12 and 13). This requirement for UTP was evident even when the added gRNA contained an oligo(U) tail synthesized during T7 transcription (lanes 8, 9, 14 and 16).

Both the gRNA-independent U insertion activity with the ND7.1x substrate and the gRNA-dependent U insertion activity with the ND7.2x substrate plus gND7x[+3] gRNA required $MgCl_2$ (data not shown) and were inhibited above 50–100 mM KCl (Figure 3).

Heparin selectively inhibits the gRNA-independent U insertion activity

A low concentration (5 μg/ml) of heparin selectively inhibited the gRNA-independent U insertion reactions with both the ND7.1x and ND7.2x substrates (Figure 4), but had little effect on the U insertion signals obtained in the presence of added gND7x[+3] gRNA, both in terms of the predominant guided band and the background ladder. Higher levels of heparin (50–100 μg/ml) inhibited both the gRNA-independent and gRNA-dependent U insertion activities (data not shown). Control experiments using uniformly labeled synthetic gRNA showed that 3′ end addition of U residues by the TUTase activity in the extract was unaffected by heparin at 5 μg/ml but was inhibited by heparin at 100 μg/ml (data not shown).

Both the gRNA-independent and the gRNA-dependent U insertion activities require ATP hydrolysis at the α–β bond

Both the gRNA-independent (Figure 5A) and gRNA-dependent (Figure 5B) U insertion activities were inhibited ~70% by the omission of ATP from the reaction. Substitution of ATP with an ATP analog (AMP-CPP) which is not hydrolyzable at the α–β bond led to almost complete inhibition of both activities, whereas substitution with an analog (AMP-PNP) which was not hydrolyzable at the β–γ bond only produced a 10–20% inhibition of both activities. Removal of GTP from the reaction had little effect on either activity (data not shown). A summary of the data from several experiments is shown in Figure 5C.

Effect of blocking the 3′ OH of the gRNA

Figure 6 shows a comparison of the U insertion activity obtained using gRNA which had been 3′ end-blocked by periodate treatment with that obtained using untreated gRNA. Control experiments showed that 70% of the added gRNA retained the 3′ block after the incubation (data not shown). The lack of a significant effect on U insertion activity suggests that the 3′ OH of the gRNA is not required for this activity.

Discussion

We have analyzed the *in vitro* insertion of Us into the first editing site of a trypanosome mRNA which has an artificial anchor sequence. The data demonstrate that the predominant number of Us inserted is specified by the number of complementary guiding nucleotides in a gRNA which has an anchor sequence complementary to the mRNA substrate, as predicted by the gRNA hypothesis (Blum *et al.*, 1990). Incubation of ND7.2x mRNA with the cognate gND7x(+7) gRNA containing the natural seven guiding nucleotides for editing site 1 resulted in the prominent insertion of seven Us into site 1 together with a minor background ladder of up to 13 U insertions. On incubation with cognate gRNAs having shorter guiding

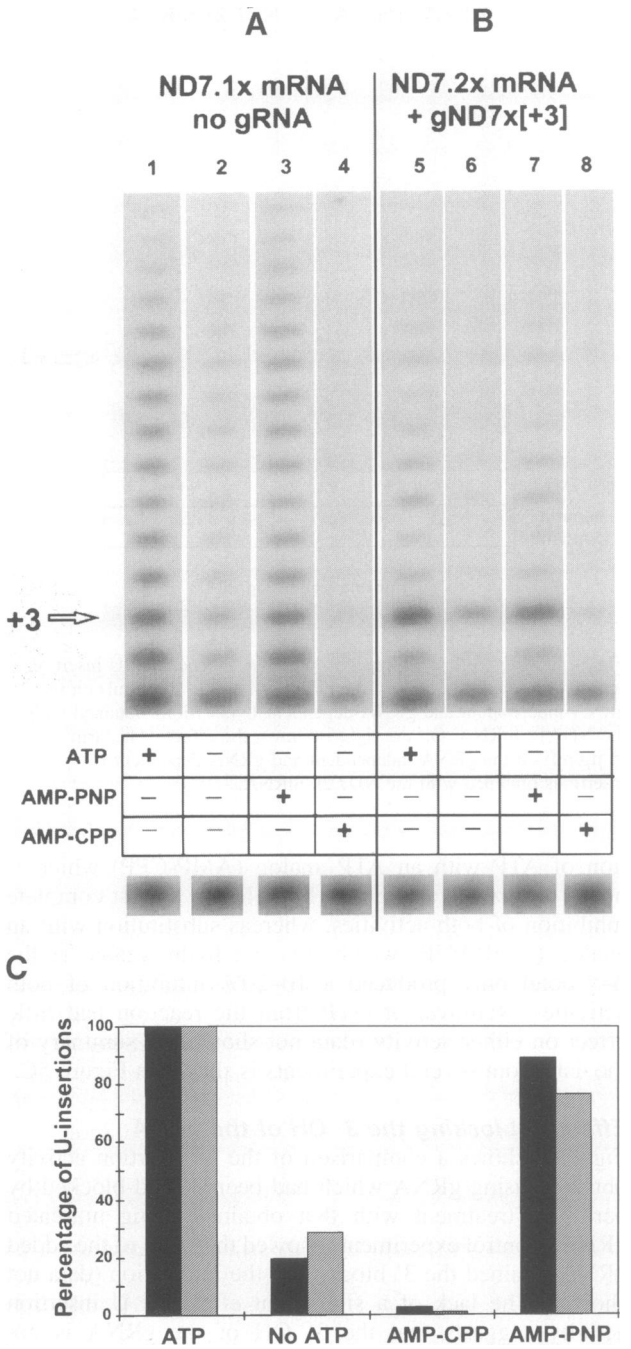

Fig. 5. ATP requirement of U insertion activities. (**A**) gRNA-independent U insertion activity using the ND7.1x mRNA substrate. ATP, AMP-CPP and AMP-PNP were added to the reactions at 1, 0.7 and 1 mM, respectively, as indicated. (**B**) gRNA-dependent U insertion activity using the ND7.2x mRNA substrate and the cognate gND7x[+3] gRNA. ATP, AMP-CPP and AMP-PNP were added to the reactions at 1, 0.7 and 1 mM, respectively, as indicated. (**C**) Summary of results from three independent experiments. Signals were quantitated by PhosphoImager analysis and were corrected for loading by the normalization signals (data not shown). The black bars indicate the gRNA-independent signals from 1–10 U insertions, and the hatched bars represent the gRNA-dependent signal for three U insertions. The ordinate is the percentage of the signal compared with the signal with added ATP as 100%.

sequences of +4 and +3 purines, the predominant insertions were of four and three Us, respectively, while gND7x[+9] gRNA, which has a guiding sequence longer than wild-type, guided the insertion of nine Us into site 1.

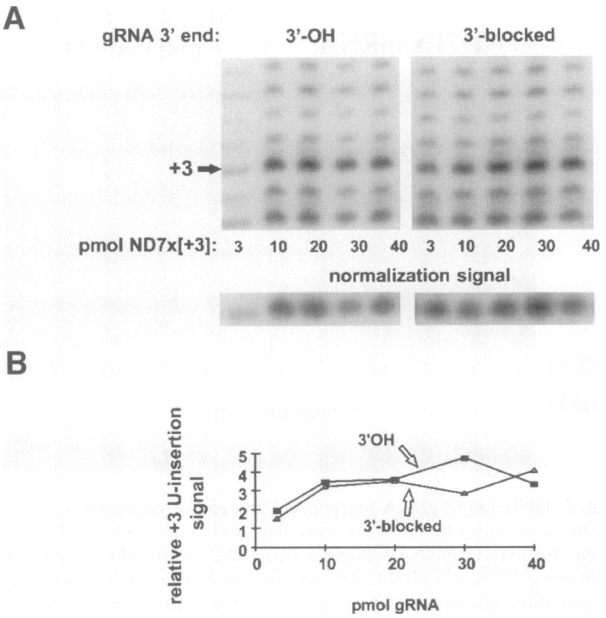

Fig. 6. Lack of effect of 3′ end blocking of added gRNA on gRNA-dependent U insertion activity. (**A**) ND72.x mRNA was incubated in mitochondrial lysate for 20 min with untreated gND7x[+3] gRNA (on the left) or gND7x[+3] gRNA which had been 3′ end-blocked by oxidation of the 3′ OH by treating with sodium periodate (on the right). The amount of added gRNA was varied. Only the lower portion of the ladders is shown. (**B**) Graph of normalized signals representing the insertion of three Us. The +3 bands were quantitated by PhosphoImager analysis. Elongation of uniformly labeled synthetic gRNA by poly(A) polymerase was employed to check the efficiency of blocking and to determine the extent to which the gRNA retained a blocked 3′ end after incubation in the mitochondrial lysate. Approximately 30% of the input RNA could be elongated after 20 min incubation in the presence of lysate compared with <2% prior to incubation (data not shown), indicating that 70% of the input RNA retained the 3′ end block after the *in vitro* reaction.

A ladder of gRNA-independent site 1 U insertions was observed with the ND7.1x and ND7.2x mRNAs *in vitro*, but not with the 3′-truncated ND7.4x mRNA. This is consistent with the hypothesis of Connell *et al.* (1996) that an intramolecular helix formed by complementary upstream and downstream mRNA sequences in CYb mRNA is required for gRNA-independent activity, and imperfectly mimics the gRNA–mRNA anchor helix, which may represent a target for RNA binding proteins involved in editing. If such intramolecular mRNA helices interact with lower affinity with the editing machinery, this could explain the sensitivity of the guide-independent activity to competition with low concentrations of heparin. The guide-independent ladders are also clearly suppressed in the presence of a gRNA lacking guiding nucleotides for site 1, and this can be interpreted as being due to disruption of the mRNA secondary structure by hybridization of the gRNA, and protection of site 1 from guide-dependent activity by the anchor, which is effectively extended by one base. As in the case of the *in vitro* gRNA-independent U insertion editing of cytochrome b mRNA substrates, it is not clear if this activity is biologically relevant *in vivo* (Connell *et al.*, 1996). There is, however, evidence that the activities mediating gRNA-independent *in vitro* U insertion and the gRNA-dependent *in vitro* U insertion/deletion are related. The *L.tarentolae* gRNA-independent U insertion activity (Frech *et al.*, 1995) and the *T.brucei*

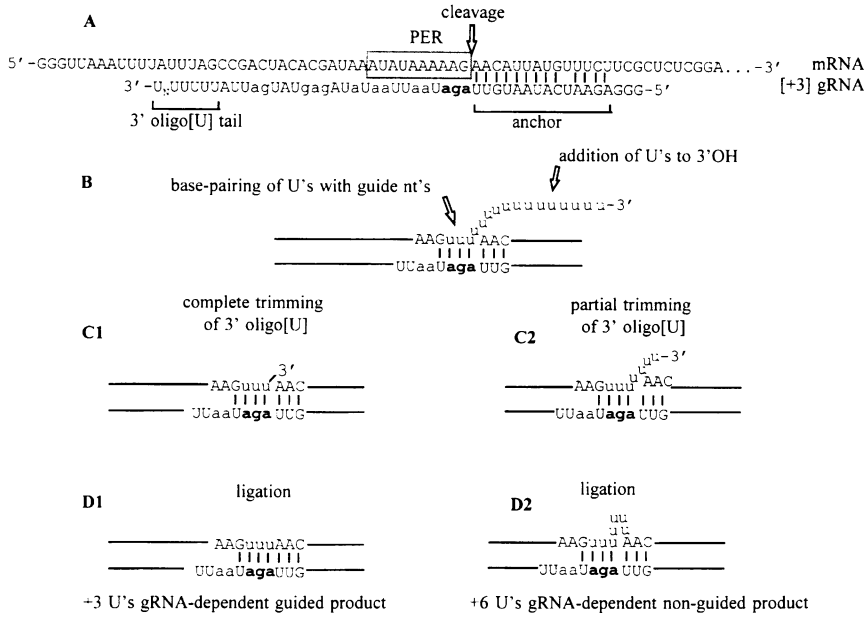

Fig. 7. Diagram of model for U insertion, using the ND7 situation. (**A**) Specific cleavage at the first mismatched base in the pre-edited mRNA, giving rise to a 3′ OH on the 5′ fragment and a 5′ phosphate on the 3′ fragment. (**B**) The U insertion activity adds a variable number of U residues to the 3′ end of the 5′ fragment. Us base-pair with the +3 guiding nucleotides. In this example, base pairing occurs adjacent to the encoded nucleotides, yielding a 3′ overhang, since the pairing can be extended by encoded upstream nucleotide(s) and since helix formation near the end of a polynucleotide chain is less stable than internal helix formation (Turner and Bevilacqua, 1993). (**C1**) or (**C2**) Complete or partial trimming of the 3′ oligo(U) overhang occurs by either a U-specific 3′ exonuclease activity or a single-strand endonuclease activity. (**D1**) or (**D2**) Ligation of the 5′ fragment to the 3′ fragment, giving rise to either an mRNA correctly edited at site 1 or an mRNA with extra Us at site 1. Molecules with too many or too few Us could also be generated by complete trimming of the oligo(U) overhang in structures which involve base pairing of the oligo(U) tail in other registers.

gRNA-dependent U deletion activity (Seiwert and Stuart, 1994; Seiwert *et al.*, 1996) both sediment at ~20S in glycerol gradients and both require α–β bond hydrolysis of ATP. As shown above, the *L.tarentolae* gRNA-dependent activity also requires α–β bond hydrolysis of ATP. Both *in vitro* activities in *L.tarentolae* extracts are inhibited above 50 mM KCl and require Mg^{2+}. The major difference between these activities is heparin sensitivity.

It is of some interest that in the presence of added gND7x[+3] gRNA, a background U insertion ladder was obtained with all three mRNA constructs, including the ND7.4x substrate mRNA which does not undergo significant guide-independent insertion (Figure 2A, lane 18). The data in Figure 4 show that the background ladders for ND7.1x and ND7.2x were not reduced substantially by 5 μg/ml heparin. This evidence suggests that the mechanism for the creation of the background ladder in the presence of added cognate gRNA containing guiding nucleotides for site 1 editing differs from the mechanism for the creation of the apparently similar ladders obtained in the absence of added gRNA. We speculate that the gRNA-dependent heparin-resistant background ladder of U insertions observed with all the mRNA substrates tested may be an integral part of the mechanism of editing. We suggest that multiple Us are first added to the 3′ end of the mRNA 5′ cleavage fragment in a template-independent fashion, and that the oligo(U) 3′ overhang not base paired to guiding nucleotides in the gRNA is then removed by either a 3′–5′ exonucleolytic or a single strand-specific endonucleolytic process identical to that occurring in the gRNA-dependent U deletion reaction (Figure 7). The efficiency of the ligation of precisely trimmed mRNA 5′ fragments to the 3′ mRNA fragment *in vitro* can be visualized by the ratio of the +3 guided U insertion band in Figure 4 to the heparin-resistant gRNA-dependent background ladder. The existence of a minor population of religated mRNA fragments containing greater or less than +3 inserted Us at site 1 could represent an alternative to misguiding by non-cognate gRNAs (Sturm *et al.*, 1992) for the generation of some misedited sequences observed *in vivo*.

Blocking the 3′ OH of added synthetic gND7x[+3] gRNA by periodation (Goringer *et al.*, 1984) had little effect on the gRNA-dependent U insertion reaction with the ND7.2x mRNA substrate This differs from the result obtained by Seiwert *et al.* (1996) for a gRNA-dependent U deletion reaction in a *T.brucei* mitochondrial extract, in which blockage of the 3′ OH of the gRNA was found to inhibit the *in vitro* U deletion activity. The reason for this is not known, but may possibly reflect either a difference in the U deletion and U insertion mechanisms or a species difference. Our results are inconsistent with models involving a transfer of Us from the 3′ oligo(U) tail of the gRNA via chimeric intermediates initiated by attack of the gRNA 3′ OH on the editing site. On the other hand, guided insertion does require added UTP and α–β bond hydrolysis of ATP. These data are most consistent with an enzymatic mechanism involving cleavage at the editing site, 3′-terminal addition of U residues derived from UTP and religation of the mRNA fragments (Blum *et al.*, 1990).

In conclusion, our analysis of the *in vitro* U insertion reaction at a single editing site demonstrates that the U insertions are specified by the guiding nucleotides in the cognate gRNA and is consistent with UTP being the

source of the inserted U residues. The availability of this *in vitro* system opens the door to a biochemical fractionation of RNA editing and a determination of the precise molecular events involved in this process.

Materials and methods

Oligodeoxynucleotides

The oligonucleotides used in these studies are listed below together with a brief description of their function: FP = forward or 5′ PCR primer, RP = reverse or 3′ PCR primer. The locations of the underlined sequences in the *L.tarentolae* maxicircle sequence (GenBank entry LEIKPMAX) are indicated. S244: TAATACGACTCACTATAGGGTTATGGTAATTAGTTACAGTG (ND7.1x mRNA FP, nt 3240–3260 in LEIKPMAX). S245: CCTCGATGTAAATACCCAATAATTAC (ND7.1x/ND7.2x mRNA RP, nt 3426–3451 in LEIKPMAX). S804: AAAAAAAAAAAAAAAAATATATTTTCTCATGTTA (PCR RP for gCYb-II gRNA transcription template, nt 2242–2291 in LEIKPMAX). S805: TAATACGACTCACTATAGGGCTTTTCTAAATAATAAAAAAAG (PCR FP for gCYb-II gRNA transcription template, nt 2242–2291 in LEIKPMAX). S1206: TAATACGACTCACTATA (T7 RNA polymerase promoter). S1529: TAATACGACTCACTATAGGGTTAAATTTTATTTAGCCGACTACACG (ND7.2x mRNA FP, nt 3286–3311 in LEIKPMAX). S1588: CTACACGATAAATATAAAAAGAACATTATGTTTCTTCGCTCTCGGACCCCAGCATCC (anchor mutagenesis of ND7 FP, nt 3305–3361 in LEIKPMAX). S1589: GGATGCTGGGGTCCGAGAGCGAAGAAACATAATGTTCTTTTTATATTTATCGTGTAG (anchor mutagenesis of ND7 RP, nt 3305–3361 in LEIKPMAX). S1679: TCCGAGAGCGAAGAAACATAATGTT (reverse transcription and extension assay of ND7x mRNAs). S1681: AAAAAAAAAAAAAATAATCATACTCCATATTTATTAATTATTTTTCTAACATTATGATTCTCCCTATAGTGAGTCGTATTA (transcription of gND7x gRNA, nt 349–395 in LEIKPMAX). S1732: AAAAAAAAAAAAAATAATCATACTCCATATTTATTAATTATTTTTCTACACTTGTAAAAATCCCTATAGTGAGTCGTATTA (transcription of gND7 gRNA). S1750: AAAAAAAAAAAAATAATCATACTCCATATTTATTAATTATCTAACATTATGATTCTCCCTATAGTGAGTCGTATTA (transcription of gND7x[+3] gRNA). S1751: AAAAAAAAAAAAAATAATCATACTCCATATTTATTAATTAAACATTATGATTCTCCCTATAGTGAGTCGTATTA (transcription of gND7x[+0] gRNA). S1855: TAATCATACTCCATATTTATTAATTATTTTTCTAACATTATGATTCTCCCTATAGTGAGTCGTATTA (transcription of gND7x gRNA lacking a 3′ oligo(U) tail). S2009: TAATCATACTCCATATTTATTAATTATCTAACATTATGATTCTCCCTATAGTGAGTCGTATTA (transcription of gND7x[+3] gRNA lacking a 3′ oligo(U) tail). S2056: CACGATAAATATAAAAAGttttttAACATTATGTTTCTTCGC [mutagenic forward primer for ND7 with site 1 edited (lower case ts), nt 3308–3343 in LEIKPMAX]. S2057: CACGATAAATATAAAAAGttttttAACATTATGTTTCTTCGC [mutagenic reverse primer for ND7 with site 1 edited (lower case ts), nt 3308–3343 in LEIKPMAX]. S2079: CCCTATAGTGAGTCGT (normalization primer for ND7x primer extension experiments, antisense to T7 promoter sequence in S1529). S2088: TAATCATACTCCATATTTATTAATTATTCTAACATTATGATTCTCCCTATAGTGAGTCGTATTA (transcription of gND7x[+4] gRNA lacking 3′ oligo(U) tail). S2257: AAAAAAAAAAAAATAATCATACTCCATATTTATTAATTATTTTTTTCTAACATTATGATTCTCCCTATAGTGAGTCGTATTA (transcription of gND7x[+9] gRNA).

mRNA and gRNA transcripts

Modified-anchor ND7 mRNAs were transcribed using T7 RNA polymerase from recombinant PCR products constructed as follows. Separate amplifications of kinetoplast DNA were carried out using S244 with S1589, and S1588 with S245. The products were then gel purified and mixed together in a second PCR reaction with more S244 and S245 to generate ND7.1x. To produce ND7.2x, S1529 was used in place of S244. ND7.2x[+7], the ND7.2x derivative edited at site 1 (+7 Us), was made by performing PCR reactions using ND7.2x as template and the primer pairs S1529 + S2057 and S2056 + S245 in separate reactions, and then combining the products with more S1529 and S245 in the second step. The ND7.4x mRNA was identical to ND7.2x mRNA upstream but had a 100 nt 3′ truncation. The ND7.4x template was synthesized by PCR using S1529 as 5′ primer and S1679 as 3′ primer.

The gRNAs were transcribed directly from synthesized oligonucleotide templates or, in the case of gCYb-II, from a PCR product, as previously described (Milligan and Uhlenbeck, 1989). Uniformly labeled gRNA was prepared by including [α-^{32}P]dNTP in the transcription reaction.

All RNAs were gel purified prior to use in the assay, and sequencing of the transcripts was performed to confirm their sequences (data not shown). Cloning and sequencing of several gRNAs transcribed with a 3′ oligo(U) tail showed that most of the molecules had the predicted 3′ ends.

Preparation of 3′-blocked gRNA by periodate oxidation

gRNA (1000 pmol) was incubated for 2 h at 4°C in the dark with 100 nmol of sodium *m*-periodate in 0.1 M sodium acetate (pH 4.5) in a volume of 1 ml (Goringer *et al.*, 1984). The RNA was collected using the RNaid kit (BIO 101) according to the manufacturers instructions and was purified on a urea–acrylamide gel.

Determination of the proportion of 3′ OH and 3′-blocked RNA

Elongation of uniformly labeled synthetic gRNA by poly(A) polymerase was employed to measure the efficiency of 3′ end blocking of labeled gRNA, and to determine the extent to which the gRNA retained a blocked 3′ end after incubation in the mitochondrial lysate. Using 0.5 pmol of uniformly labeled periodate-treated or untreated control gRNA, or 1/30 of the RNA extracted after incubation with lysate, as above, incubation was carried out for 60 min at 37°C in a volume of 5 µl in 50 mM Tris (pH 7.8), 10 mM $MgCl_2$, 250 mM NaCl, 2.5 mM $MnCl_2$, 1 mM ATP, 0.35 mg/ml bovine serum albumin (BSA), with 0.6 U poly(A) polymerase (Pharmacia). The reaction was stopped with the addition of 16 µl of 10 mM EDTA, and the RNA then collected by phenol extraction and analyzed by electrophoresis on an 8% polyacrylamide–8 M urea gel. The ratio of 3′ end-elongated to non-elongated (blocked) RNA was quantitated using a PhosphoImager (Molecular Dynamics). The results indicated that <2% of the periodate-treated RNA remained unblocked. After incubation with the mitochondrial lysate, the percentage of unblocked RNA increased to ~30%, probably as a result of nibbling of the 3′ ends by nuclease activity in the lysate.

PCR

PCR was performed in 100 µl reactions containing 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 2.5 mM $MgCl_2$, 1 mg/ml BSA, 200 µM of each dNTP, 0.5 µM of each primer and 2.5 U of *Taq* polymerase (Perkin Elmer). The PCR profiles were 5 min at 95°C followed by 30 cycles of 95°C for 30 s, 55°C for 30 s or 60 s, and 72°C for 60 s.

Isolation of mitochondria

Leishmania tarentolae (UC strain) cells were grown to a late log phase density of 10^8 cells/ml as previously described (Simpson and Braly, 1970). After washing twice in 0.25 M sorbitol, 20 mM HEPES, 2 mM EDTA (pH 7.5) (SHE), the cells were resuspended in this isotonic medium at a density of 10^9 cells/ml and disrupted in a Stansted Power Fluid apparatus at 60–65 p.s.i. The crude mitochondrial fraction was pelleted, resuspended in 0.25 M sorbitol, 20 mM HEPES (pH 7.5), 2 mM $MgCl_2$, digested with DNase I to remove contaminating nuclear DNA, and washed by centrifugation in SHE. The crude fraction was taken up in 76% Renografin and a purified mitochondrial fraction isolated by flotation in Renografin density gradients as described previously for hypotonically broken cells (Braly *et al.*, 1974), and resuspended in 20 mM HEPES pH 7.5, 10% glycerol, and 20 mM KCl for storage at –80°C. After thawing on ice, the mitochondria were lysed by addition of Triton X-100 to a final concentration of 0.3%

Lysate-mediated reactions

In a 20 µl volume containing 12 mM HEPES (pH 7.5) and 0.1 mM EDTA, 3 pmol of the T7-transcribed mRNA construct were added to 30 pmol of the appropriate gRNA and denatured at 65°C for 3 min. The RNA solution was adjusted in a 50 µl volume to a final concentration of 5 mM HEPES (pH 7.5), 0.04 mM EDTA, 20 mM KCl, 3 mM potassium phosphate (pH 7.5), 10 mM $MgCl_2$, 20 mM dithiothreitol (DTT), 2 mM spermidine, 1 mM ATP, 1 mM GTP and 1 mM UTP. Reactions were initiated by the addition of ~10^9 cell equivalents of the mitochondrial lysate containing ~30 µg of protein. After incubation at 27°C for 40–60 min, reactions were terminated by incubation with proteinase K and SDS for 15 min at 37°C prior to phenol–chloroform extraction.

Primer extension assay

ND7.1x and ND7.2x mRNAs which had been incubated in the mitochondrial lysate were reverse transcribed using AMV reverse transcriptase

and the S1679 oligonucleotide (3 pmol). The reactions were terminated by heating at 90°C for 5 min, then the nucleic acids were precipitated with ethanol and mixed with excess (90 pmol) of the S1529 oligonucleotide, a portion of which was 5′-^{32}P-labeled, and a PCR reaction performed (30 cycles, as described above). The reactions were phenol extracted and the products, which represent mainly single-stranded DNA sense copies of the RNA template, were purified by electrophoresis in a 16 cm 8% polyacrylamide–8 M urea gel. A gel slice extending 5 mm above the labeled extension band was recovered to ensure that sequences having inserted bases were collected.

The DNA samples were assayed for U (=T) insertions in editing site 1 by extending the primer S1679 with [α-^{32}P]dATP using Sequenase (U.S. Biochemical). A normalization primer, S2079, was used to control for variations in the amount of template. Since the lysate-mediated U insertions occur infrequently, the signal intensity from the normalization primer is much greater than that from the assay primer (S1679), and it can interfere with quantitation. For this reason, the intensity of the normalization extensions was decreased by using a mixture of 3′-blocked S2079 (ddT was added using terminal transferase, Boehringer Mannheim) with a small amount of untreated normalization primer. Assay reactions were performed at 50°C for 1 h and contained 1 pmol of sample DNA template, 10 pmol of assay primer S1679, 0.2 pmol of the normalization primer S2079, 10 pmol of S2079 blocked at the 3′ end with ddT as described above, 100 mM NaCl, 20 mM $MgCl_2$, 40 mM mM Tris–HCl (pH 7.5), 4 mM DTT, 0.625 μM [α-^{32}P]dATP (800 μCi/mmol) and 1 U of Sequenase. The template and oligos were first heated at 65°C for 3 min, then annealed for 3 min at 50°C, together with the NaCl, $MgCl_2$ and Tris in a volume of ~8 μl, before addition of the [α-^{32}P]ATP and enzyme. ddCTP was included at 0.125 mM in some reactions. Reactions were terminated by addition of 7.2 μl of formamide loading buffer, prior to electrophoresis in a 12% polyacrylamide–8 M urea gel. Quantitation was performed with a PhosphoImager (Molecular Dynamics).

A linear response of the normalization extension product was observed when the DNA template was varied from 0.25 to 2 times the standard amount (data not shown). In addition, control experiments showed the +7 signal increased linearly when synthetic ND7.2x mRNA with seven Us at site 1 was mixed with pre-edited ND7.2x RNA in a 1/10 000 to 1/100 molar ratio (data not shown).

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