Substrate Determinants for RNA Editing and Editing Complex Interactions at a Site for Full-round U Insertion*

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Multisubunit RNA editing complexes catalyze uridylate insertion/deletion RNA editing directed by complementary guide RNAs (gRNAs). Editing in trypanosome mitochondria is transcript-specific and developmentally controlled, but the molecular mechanisms of substrate specificity remain unknown. Here we used a minimal A6 pre-mRNA/gRNA substrate to define functional determinants for full-round insertion and editing complex interactions at the editing site 2 (ES2). Editing begins with pre-mRNA cleavage within an internal loop flanked by upstream and downstream duplexes with gRNA. We found that substrate recognition around the internal loop is sequence-independent and that completely artificial duplexes spanning a single helical turn are functional. Furthermore, after our report of cross-linking interactions at the deletion ES1 (35), we show for the first time editing complex contacts at an insertion ES. Our studies using site-specific ribose 2′-hydroxyl at ES2 is essential for cleavage but dispensable for editing complex cross-linking. This study provides new insights on substrate recognition during full-round editing, including the relevance of secondary structure and the first functional association of specific (pre-mRNA and gRNA) ribosides with both endonuclease cleavage and cross-linking activities of editing complexes at an ES. Importantly, most observed cross-linking interactions are both conserved and relatively stable at ES2 and ES1 in hybrid substrates. However, they were also detected as transient low-stability contacts in a non-edited transcript.

The single-mitochondron containing kinetoplastid protozoa, including species of Trypanosoma and Leishmania, use cycles of uridylate insertion or deletion at numerous editing sites (ESs)4 with pre-mRNAs to generate mature mRNAs (for recent reviews, see Refs. 1–3). This post-transcriptional mRNA maturation is catalyzed by a multisubunit editing complex (4–8) with specificity for the ESs being directed by small trans-acting guide RNAs (gRNAs) that are partially complementary to pre-mRNA (9–12).

A significant body of information has been accumulated on the functional and structural composition of editing complexes, including the identity of the subunits catalyzing the three steps of each editing cycle; they are mRNA cleavage at deletion and insertion ESs (13, 14), U addition or U removal (15–17), and RNA ligation at deletion and insertion ESs (18–23). The complexes are heterogeneous in protein composition but share most of the approximately 20 subunits identified (24). Several factors are also known or proposed to play auxiliary roles in editing (8, 25–33), although they are dispensable in vitro (5, 6, 8, 34).

Much less is known about the mechanisms of substrate recognition including the protein subunits and substrate determinants that distinguish pre-edited (pre-) mRNAs from other transcripts and DNA in mitochondria. We recently reported the first observations of direct editing complex interactions with a functional site for full-round U deletion, showed preferential association with the editing substrate, and provided evidence for one of the interacting subunits corresponding to KREPA2 (Ref. 35). However, editing complex interactions at insertion sites have not been reported. Other recent reports showed that bacterially expressed recombinant versions of the subunits KREPA3 and KREPA4 bind RNA (36, 37). The latter exhibited specificity for a gRNA 3′ oligo(U) tail.

In pre-mRNA/gRNA substrates, unpaired pre-mRNA uridylates or unpaired gRNA purines are landmarks of deletion or insertion sites, respectively (9), and the number of such residues dictates the extent of U removal or addition (9, 10, 38). The two kinds of editing are likely to be differentially regulated as they involve separate activities and enzymes (13, 14, 18, 22, 39, 40), and there is evidence for their physical separation in heterogeneous complexes and subcomplexes (23, 24). Interestingly, efficient deletion and insertion editing have distinct requirements for a proposed pre-mRNA/gRNA ligation bridge (42, 43), and

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4 The abbreviations used are: ES, editing site; gRNA, guide RNA; nt, nucleotide(s); dsRNA, double-stranded RNA.

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artificially interconverted sites use differing pre-mRNA lengths (41). The above observations suggest that the editing complex recognitions in and near an ES may also differ between the two editing types.

Our interconversion of functional ESs from deletion to insertion and vice versa experimentally demonstrated that the basic determinants that commit editing complexes into full-cycle deletion or insertion reside within the internal loop containing the targeted ES (41). However, additional features proximal and/or distal to an ES may modulate the efficiency of editosome assembly and catalysis. For example, discrete sequence changes affecting the pairing potential of residues adjoining an ES can significantly impact the specificity and efficacy of full-round and partial (“pre-cleaved”) editing (43, 44). The current model of trypanosome RNA editing postulates that natural sites and partial (“pre-cleaved”) editing (43, 44). The current model of trypanosome RNA editing postulates that natural sites should be flanked by a proximal upstream duplex between a

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Deoxynucleoside-substituted transcripts were made by (IDT, Inc.), and 2'-F and 2'-OCH₃-modified transcripts were by (Dharmacon, Boulder, Co). Ribonucleotides are denoted by the prefix "r".

Pre-mRNA Strand—[13] GGGrGGGAGGAAGArArGrArArArArArGrArArGrArArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGrArArGr

Editing and Cleavage Assays and Quantitation Analysis

Full-round U insertion was performed as described (39). Briefly, a 2-μl mixture with pre-annealed 3'-end-labeled pre-mRNA (~10 fmol) and gRNA (1.2 pmol) was completed to 20 μl with 10 mM MRB buffer (10 mM magnesium acetate, 10 mM KCl, 1 mM EDTA, pH 8, 25 mM Tris-HCl, pH 8, and 5% glycerol), 150 μM UTP, 3 μM ATP, and 2 μl of peak editing fraction. The reaction was incubated at 26 °C for 60 min, deproteinized, and resolved in 9% acrylamide, 7 M urea gels. Editing complexes were pre-treated with 10 mM PPⅠ, to score total cleavage in absence of RNA ligase activity (22). Neither ATP nor UTP were added to this assay, and the cleavage products were resolved in 15% PAGE with 7 M urea. Ribonuclease T1 and hydroxyl ladders were used to confirm the cleavage at ES2 (not shown). All pre-mRNAs for editing were 3'-end-radiolabeled with [32P]cytidine-3',5'-diphosphate except for the 2'-F-modified transcript (Pair-17), which had to be made with a 3'-terminal deoxynucleoside (Dharmacon). Such a terminus prevents radiolabeling with T4 RNA ligase (57), so this transcript was 5'-end-labeled with T4 polynucleotide kinase. Data were visualized by phosphorimaging and/or x-ray autoradiography, and quantitation was performed using a STORM PhosphorImager (ImageQuant 5.0, GE Healthcare). Each panel in the figures corresponds to one of two replica series performed simultaneously (i.e. one experiment). At least two independent experiments were performed for each figure, and the data shown are representative. The editing activity varied between editosome preparations, but the relative efficiency of the constructs was always consistent. The abundance of accurately edited and cleavage product for each construct tested was initially calculated as the percentage of total input RNA and then normalized to the abundance of the corresponding product by the parental Pair-1 substrate. Mean and error bars were plotted on a linear scale.

Annealing and Photo-cross-linking Assays

The efficiency of pre-mRNA/gRNA annealing was scored in native gels. ES2-radiolabeled mRNA (~10 fmol) and gRNA (1.2 pmol) were pre-annealed in a 2-μl mixture for 10 min at 37 °C followed by 10 h at 26 °C, as for editing assays. 20-μl mixtures were completed with 10 mM MRB buffer (see above) containing xylene cyanol and bromphenol blue, incubated for an additional 10 min at 26 °C, loaded directly onto a 6% native PAGE, and resolved at 180 V for 6 h at 4 °C. 0.5× Tris-buffered EDTA buffer and 1 mM MgCl₂ were used in both the gel and running buffer. The photo-cross-linking assays were performed using pre-annealed RNA pairs and under editing reaction conditions (but without nucleotides) as recently reported (35). Also, co-immunoprecipitation and competition analyses were carried out as described (35). All competitor transcripts were supplemented to mixtures and incubated for extra 10 min at 26 °C to
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**Analysis of the Natural A6 Pre-mRNA Features Proximal to ES2 for Full-round Insertion**—Features in the RNA substrate that are specifically recognized during full-round editing are not fully defined in trypanosomes. These may include the native pre-mRNA sequence, purine richness, and higher-order structure of the pre-mRNA/gRNA bimolecular substrate. To address this question we characterized the proximal features of the pre-mRNA/gRNA-paired residues that are specifically recognized during full-round editing (Fig. 1). We have shown that minimal 43–45-nt pre-mRNA substrates support efficient full-round insertion in the A6, CYb, and RPS12 systems (41). For simplicity, we will refer to the upstream and downstream duplexes (relative to the pre-mRNA) flanking the internal loop containing ES2 as “left” and “right" helices. The terminal 5’ extensions of Pair-1 will be termed pre-mRNA and gRNA protrusions, respectively (Fig. 1A).

We first analyzed the left helix of Pair-1. Our previous studies showed that virtually all natural 5’ purines in the pre-mRNA protrusion could be substituted by pyrimidines (41). It was also reported that natural pre-mRNA sequence beyond the residues forming the right duplex were dispensable for ES2 insertion (47). To assess the importance of the natural pre-mRNA sequence in the left helix and the requirement for the pre-mRNA protrusion, we designed Pair-2 containing an artificial 15-bp blunt-ended left duplex (Fig. 1A). Furthermore, the pre-mRNA/gRNA-paired residues in this duplex were flipped to alternate all purines and pyrimidines (except the first two residues needed for T7 in vitro transcription). Pair-2 supported insertion at a level comparable with the parental Pair-1 (Figs. 1, B and C), thus showing that neither the natural pre-mRNA sequence nor purine richness in the left duplex nor the pre-mRNA protrusion is required for efficient insertion. Note that to preserve both a pre-mRNA functional length (41) and its purine content in Pair-1 (77.8%), all 25 upstream purines in the parental substrate were moved to the 3’ end of Pair-2 (75% purines).

To further analyze the functional length of the left helix, we tested Pair-2 derivatives (Fig. 1A) containing 12-bp (Pair-3) or 11-bp (Pair-4) helices with predicted stabilities similar to the parental duplex (data not shown). Interestingly, Pair-3 was edited less efficiently than Pair-1. This may reflect a partial influence of nucleotide composition of the helix. Notably, the artificial 11-bp upstream duplex in Pair-4, which represents ~one turn of helical RNA (58), efficiently replaced the complete 5’ region of the parental Pair-1.

Because the minimal length of the natural A6 pre-mRNA for efficient full-round ES2 insertion is ~43–45 nt (41), we trimmed the rightward region of Pair-4 to generate Pair-5 (45-nt pre-mRNA; Fig. 2A). This substrate, with reduced purine content (71%), supported less accurate editing (i.e. 3U addition) than the parental Pair-1 and accumulated inaccurate insertion by 1U addition (Fig. 2B). However, relocation of the protruding 3’ purines of Pair-5 to the 5’ end (Pair-6; 71% purines) re-established 3U insertion nearly to the level of the parental Pair-1 (lane 3). Although the latter 45-nt constructs imply that a short protrusion may be more stimulatory 5’ than 3’ to ES2, alternative structural reasons are also feasible. To determine whether or not the pre-mRNA extension in Pair-6 must be purine-rich, we substituted most of the protruding purines by pyrimidines (Pair-7). Interestingly, Pair-7 was about as efficient as Pair-1 despite its relatively low (49%) purine content (Fig. 2C, compare the first and last lanes). Together, the above results indicate that neither the natural polypurine run nor the overall purine richness of A6 pre-mRNA is a critical determinant of ES2 insertion in vitro. Combined with our previous observations (41), these
results suggest that the recognition of the A6 pre-mRNA features upstream of the internal loop is sequence-independent. Furthermore, a single turn of helical RNA was sufficient for full-round insertion. Additional work will be required to test whether smaller and less stable left helices are efficient.

We then examined the features of the right helix of Pair-1 required for editing. To this end we used variants of Pair-6 (Fig. 2A) containing 14-bp (Pair-8) and 13-bp (Pair-9) right duplexes with a predicted stability comparable with the parental helix (data not shown). Both Pair-8 and Pair-9 supported editing nearly as efficiently as the parental Pair-1 (Fig. 2C). We then tested derivatives bearing either 12-bp (Pair-10) or 11-bp (Pair-11) right helices. Notably, whereas the base composition of the 3′ duplex significantly deviated from the parental helix, these substrates were appreciably more efficient than Pair-1 (Figs. 2, D and E). It is also worth noting that the predicted stability of the right helix in Pair-11 is ~10% lower than in Pair-1 (see the legend to Fig. 2). Finally, we generated Pair-12 bearing a blunt-ended right duplex of 11 bp, and a change of the 5′-terminal two gRNA residues to facilitate in vitro transcription starting with G (54). This last construct was less efficient than Pair-11. It is possible that a branch structure downstream of ES2 favors insertion editing. Consistent with this notion, a derivative of Pair-10 with a right blunt-ended duplex was also less efficient (data not shown).

Altogether, these data show that substrates with ~one turn of helical RNA at both the left and right sides of ES2 support efficient full-round U insertion and that the natural sequence of the minimal (~43–45 nt) pre-mRNA outside the internal loop is not required. This suggests that the basic editing complex recognizes flanking the internal loop involve sequence-independent features of the pre-mRNA/gRNA pair.

**Effect of Ribose 2′-Substitutions on Full-round Insertion**—We analyzed the contribution of ribose 2′-hydroxyl groups to substrate recognition by editing complexes, by incorporating 2′-deoxy substitutions and other 2′ modifications in and around ES2 in the parental Pair-1 (summarized in Fig. 6).

**pre-mRNA Residues Upstream of the Internal Loop**—We first analyzed the pre-mRNA 5′ region (Fig. 3A) using derivatives containing 11 deoxynucleotides that replaced either the entire pre-mRNA protrusion (Pair-13) or most bases in the left helix (Pair-14). Interestingly, both constructs supported insertion at about half the level of the parental Pair-1 (Fig. 3, upper panel). This decrease in editing was largely paralleled by a reduction in ES2 cleavage (Fig. 3, lower panel, and C). Importantly, pre-mRNA cleavage was scored in absence of RNA ligase activity using editing complexes that were pre-treated with 10 mM PPI (Ref. 22; see “Experimental Procedures”). These two constructs showed that 2′-hydroxyl groups in the pre-mRNA protrusion and the left helix partially stimulate insertion. Our previous work showed that truncation of the protrusion in the minimal 45-nt A6 pre-mRNA (see 34-nt RNA8 in Cruz-Reyes et al. (41)) abrogates full-round insertion. This suggests that editing complexes make sufficient contacts with the all-DNA protrusion to support an appreciable insertion level, and thus, the 2′-hydroxyls of the pre-mRNA protrusion are significantly stimulatory but not essential. Furthermore, the left RNA/DNA heteroduplex in Pair-14 should adopt a shape that is more similar to the A-form (of dsRNA) than the
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A

RNA pair

1. GGGGAGGAAGACGAGAGGAGGAGUCAUGAAAAGGUAAUG
   UUCUUCUCCCUUUUG UAGGCCUAAUAUGG AAAGAA

B

Pair -g 1 13 14 15 16

Input

C

Relative product

-0.1 0.1 0.5 1.0

D

Pair -g 1 17

Cleaved

E

G

Input

G

Relative product

-0.1 0.1 0.5 1.0

FIGURE 3. Ribose 2'-substitution of upstream and downstream residues in the pre-mRNA of Pair-1.

A, Pair-1 and derivatives are aligned (only the pre-mRNA strand is shown). Single or multiple 2'-substitution s are indicated in boxes. The modifications are 2'-deoxy (-H), 2'-methoxy-(OCH3) or 2'-fluorine (-F). The latter substrate had to be manufactured with a deoxynucleoside at the 3'-end (empty box). All other labeling is as in Fig. 1. B, insertion and cleavage assays (upper and lower panels, respectively) of 5'-substituted pre-mRNAs. A control lane devoid of gRNA (-g) was included. C and G, plots of relative accumulation of both accurately edited (by 3U addition; black bars) and ES2 cleavage (white bars) using 3'-end radiolabeled pre-mRNA. D, cleavage assay of 2'-fluorine modified. This pre-mRNA was 5'-end radiolabeled (see "Experimental Procedures"). Precise cleavage at ES2 was confirmed using ribonuclease T1 RNase and hydroxyl partial pre-mRNA digestions (not shown (46)). E, editosome photo-cross-linking with pre-mRNAs containing a single 32P and 4-thioU at ES2. A diagram of thiolated Pair-1 (Pair-1); above) indicates the labeled bond (*) and thiolated U (g). Note that the right duplex was extended by one base pair between Thio-U and a guiding adenosine. ES-2 cross-linking of thiolated Pair-1 and Pair-15 with editosome components (below). The size of molecular markers is indicated in kDa. F, insertion and cleavage assays of 3'-substituted pre-mRNAs.

B-form (of dsDNA; Ref. 59), so the decrement in insertion could mainly reflect a requirement for 2'-hydroxyls, although shape-dependent recognitions may also be important.

We then tested the effect of a single 2'-deoxynucleoside substitution adjoining the scissile bond (Pair-15). Notably, this 2'-H abolished both insertion and cleavage (Figs. 3, B and C). This may reflect a direct role of the 2'-OH at ES2 in catalysis and/or site recognition or an indirect role due to a change in the sugar pucker (from ribose C3'-endo to deoxyribose C2'-endo conformation (58)). To address these two possibilities we tested other ribose 2'-modifications such as 2'-O-methyl (OCH3; Pair-16) that favor the RNA-like sugar pucker but cannot act as a hydrogen bond donor (57). 2'-OCH3 supported neither insertion nor cleavage (Figs. 3, B and C), but it is conceivable that the added bulk of this group, compared with a 2'-OH, caused steric hindrance. We then tested the smaller 2'-fluorine (F) modification (Pair-17), which should also promote an RNA-like C3'-endo conformation even more than 2'-OCH3 and is highly unlikely to accept a proton (57). This substitution also completely inhibited cleavage (Figs. 3, B and C; see the legend). Unfortunately, a 2'-NH2 modified guanosine at ES2 is not available (Dharmacon).6 Based on these results, it is conceivable that formation of a hydrogen bond by the ribose 2'-OH group at ES2 is required for insertion.

The ribose 2'-hydroxyl group at ES2 could mediate either catalysis at pre-mRNA cleavage, editing site recognition, or editing complex assembly onto the substrate. To attempt distinguishing between these possibilities, we modified our recently developed photo-cross-linking assay to analyze direct editing complexes at ES1 in A6 pre-mRNA (35). To similarly assay ES2, we placed a single 32P-labeled 4-thioU immediately 3' of the scissile bond. Pairing of this residue with a guiding adenosine should extend the right helix by 1 base pair (Pair-15'; Fig. 3E). Notably, the thiolated ES2 supported protein cross-linking interactions that both are similar to those reported for ES1 (35) and specifically co-purify and co-immunoprecipitate with editing complexes (see Fig. 7). Substrates with either a 2'-OH (Pair-15) or the inhibitory 2'-H modification (Pair-15') at ES2 exhibited identical cross-linking patterns (Fig. 3E), suggesting a similar editing complex association with both the 2'-H-substituted and unmodified ES2. Thus, the single 2'-deoxy substitution at ES2 does not seem to prevent editing complex interactions at ES2,

6 Dharmacon, personal communication.
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with Pair-1 (Figs. 3, F and G). This inhibition increased with the extent of deoxy substitution. Notably, Pair-20 with all upstream pre-mRNA residues modified was most inhibited. To determine the importance of the 2'-hydroxyl immediately 3' of the cleavage site, the singly substituted Pair-21 was tested. This substrate was about half inhibited in both editing and cleavage assays (Figs. 3, F and G). Combined, these observations indicate that the 2'-hydroxyls just 3' of ES2 and further downstream in the duplexed pre-mRNA are significantly stimulatory. These deoxy substitutions did negatively impact insertion, primarily at pre-mRNA cleavage.

gRNA Residues and Duplexes Flanking the Internal Loop—Apart from the critical ribose 2'-OH at the editing site, most pre-mRNA 2'-hydroxyl groups tested were stimulatory but not essential for ES2 insertion. We then examined the effect of proximal gRNA substitutions and DNA duplexes (Fig. 4A). A 10-deoxynucleotide patch on the gRNA strand at either side of the internal loop (Pair-22 and Pair-24, respectively) had a slight negative effect on insertion (Fig. 4B) comparable with that observed with corresponding pre-mRNA patches (Pair-14 and Pair-18). In contrast, DNA duplexes formed by the complementary patches at left (Pair-23) or right (Pair-25) of ES2 were more inhibitory, particularly the Pair-25 (Fig. 4B). In both cases, insertion and pre-mRNA cleavage at ES2 were similarly inhibited (Fig. 4D). Interestingly, the right DNA duplex also significantly affected the scissile-bond selectivity. That is, the pre-mRNA of Pair-25 was cleaved at several residues flanking ES2; the upstream cuts are in the loop, whereas the downstream cuts are in the right duplex. All these cleavages were gRNA-dependent (not shown).

We assessed whether the inhibitory DNA duplexes affected the editing complex photo-cross-linking interactions with ES2. Interestingly, thiolated versions of these substrates (Pair-23' and Pair-25') reduced the level of all cross-linking subunits (Fig. 4E). This effect was particularly severe with Pair-25'. However, because protein-RNA cross-linking can be quite sensitive to conformational changes, inhibition of cross-linking activity may reflect local structural changes of the editing site rather than reduced editing complex assembly onto the RNA substrate. A native gel analysis of DNA duplex-containing pairs and Pair-1 confirmed complete annealing of these substrates (Fig. 4F). Combined, the parallel inhibition of insertion, cleavage, and cross-linking activities at ES2 suggest that proximal DNA duplexes negatively impact a productive interaction of editing complexes with the substrate and, thereby, catalysis.

Internal Loop Residues—Finally, we examined the importance of 2'-hydroxyl groups in the internal-loop residues containing ES2 (Fig. 5A). Deoxy substitutions in the three residues consistent with the possibility that the ribose 2'-hydroxyl adjoining the scissile bond may play a role in catalysis.

Pre-mRNA Residues in the Right Helix—We then examined the pre-mRNA residues in Pair-1 that are part of a 15-bp right duplex. Three deoxynucleotide patches were initially compared, 10, 14, and 15 nt long (Pairs 18, 19, and 20, respectively; Fig. 3A). Note that these pre-mRNAs contain a 3'-most ribonucleoside to allow end-radiolabeling with T4 RNA ligase (see “Experimental Procedures” (57)). Interestingly, these ribose substitutions decreased both insertion and cleavage compared

FIGURE 4. Ribose 2'-substitution of Pair-1 pre-mRNA and gRNA strands. A, pair-1 and derivatives are aligned. Internal loop residues around ES2 are depicted as curved lines with an arrowhead pointing to ES2. Deoxynucleoside substituted residues are boxed. B, insertion assay. C, cleavage assay. The inaccurate cleavages with Pair-25 are also gRNA dependent (data not shown). Plots (D) and photo-cross-linking assays (E) are as in Fig. 3, F, annealing assays of RNA pairs used in E. Control lanes without gRNA (-g) are indicated.

FIGURE 5. Ribose 2'-substitution of internal loop residues around ES2 in Pair-1. A, Pair-1 and derivatives. B, insertion assay. C, cleavage assay. ES2-cleavage products are marked with filled dots. Note that the offset gel mobility reflects the use of the same pre-mRNA in Pairs 1 and 27 as well as in Pairs 26 and 28. D, plots and E, photo-cross-linking assays are as in Fig. 3, F, annealing assays of RNA pairs used in D. Control lanes without gRNA are indicated.
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| Left | Internal Loop | Right |
|------|--------------|-------|
| Moderate (L1, L2) | Moderate (L3) | n.d. |
| n.d. | n.d. | n.d. |

(L1) pre-mRNA protrusion or either strand of the duplex.

(L2) 2’-Hs on both duplex strands inhibit more than on one strand.

(L3) Test with 2’-Hs on both strands.

(L1) Strong in combination with gRNA loop 2’-Hs.

(L2) Even more inhibitory in combination with gRNA loop 2’-Hs.

(R1) Either strand of duplex. Increased with extent of 2’-H modification.

(R2) Both strands of the duplex combined. Induced cryptic cuts.

FIGURE 6. Summary of ribose 2’-deoxy substitutions tested on Pair-1 for full-round insertion at ES2. Upper panel, indicates the level of inhibition for the left and right sides of the internal loop and within the loop (each region is separated by a vertical line). Full-round insertion and pre-mRNA cleavage (Ins/Endo) are on the left, and editosomes cross-linking (X-links) are at the right. The asterisk indicates that insertion and cleavage are affected at comparable levels. Circle types representing the level of inhibition: thick line, moderate; thin line, not determined (n.d.); gray (in addition to thick line), strong; with a pattern, no effect; black, complete. Parentheses indicate clarification notes (lower panel). A filled arrowhead points to the natural ES2 for full-round insertion. dsDNA within the right duplex induced cryptic cuts at several residues (open arrowheads) flanking the editing site. Middle panel, diagram of Pair-1 with individual residues shown as circles. Lower panel, explanatory notes on the effect of the indicated modifications.

![Diagram](Image)

5’ of the guanosine at ES2 (Pair-26) had virtually no effect on either insertion or cleavage (Fig. 5, B and C). In contrast, 2’-H substitution in all seven loop gRNA nucleotides (Pair-27) significantly reduced editing and cleavage (Fig. 5, C and D). Notably, modification of both strands of the internal loop (Pair-28) further inhibited both editing and cleavage. Furthermore, editing complex cross-linking at ES2 was also moderately and strongly reduced in the corresponding Pair-27 and Pair-28 substrates, respectively (Fig. 5E). A native gel analysis confirmed the complete annealing of these pairs (Fig. 5F).

Together, these observations indicate that several hydroxyl groups in the internal loop are relevant determinants of insertion that largely influence both the efficiency of pre-mRNA cleavage and cross-linking by editing complexes. Interestingly, hydroxyls on the gRNA strand appeared to compensate for deoxy substitutions on the pre-mRNA strand but not vice versa. That is, one or more 2’-hydroxyls in the gRNA internal loop residues significantly stimulate in trans pre-mRNA cleavage and/or site recognition by editing complexes. The analyses in Figs. 3–5 are summarized in Fig. 6.

Specificity of Cross-linking Interactions at the Insertion ES2—Several observations indicate that the protein-RNA photo-cross-linking interactions at the insertion ES2 represent direct contacts of editing complexes with the substrate. For example, the cross-links are specifically co-immunoprecipitated by antibodies raised against protein subunits of the complex (Fig. 7A and data not shown). Also, native complexes purified by either two or three steps of consecutive ion-exchange chromatography exhibit comparable cross-linking (Fig. 7B) and editing activities at ES2 in the minimal substrate analyzed (41). Thus, although the latter preparation has a simpler protein composition (Fig. 7C), the two preparations of editing complexes appear functionally equivalent with the substrate analyzed. Furthermore, the presence of representative subunits (Fig. 7D and data not shown) as well as all critical catalytic activities including editing endonucleases suggest that the functional and protein composition of our complexes is similar to that reported by other groups (1, 2). Together, these results indicate that the cross-linking interactions at ES2 are specific to editing complexes. This is consistent with our recently reported observations of cross-linking interactions by editing complexes at the deletion site ES1 in a similar A6 substrate (35).

We analyzed the specificity of the cross-linking and pre-mRNA cleavage activities at ES2 in competition analyses with homologous and heterologous transcripts (Figs. 8, A and B). In both cases the bimolecular A6 substrate was readily competed out by a 5- and 10-fold excess of homologous pre-mRNA that can hybridize with free cognate gRNA (~120 times the pre-mRNA concentration in the standard mixture; see “Experimental Procedures”). In contrast, heterologous transcripts including another pre-mRNA, tRNA, and a non-complementary gRNA were partially or not inhibitory at greater (10-
(200-fold) excess. Finally, further addition of cognate gRNA was not inhibitory. Thus, both cross-linking and cleavage activities of editing complexes at ES2 exhibit specificity for the hybrid substrate. These observations are also consistent with our reported preferential interactions of editing complexes at ES1 (35). Interestingly, the highly structured tRNA competitor partially affected both cross-linking and editing activities more at ES2 than at ES1 (Ref. 35; Fig. 8).

The similar gel mobility of mayor cross-links at ES2 and ES1 (Fig. 9) suggested that the same subunits of editing complexes make these contacts. However, the cross-linking efficiency at ES1 is significantly greater than at ES2, consistent with the former substrate supporting a much higher level of editing in vitro (42).

Because the cross-linking patterns at ES2 and ES1 are similar, we asked whether a transcript that does not undergo editing can cross-link with editing complexes. To this end we tested gRNA D33, which does not inhibit ES1 cross-linking at an ~200-fold excess and exhibits virtually no predicted structure (35). Surprisingly, such a transcript containing a single photo-reactive 4-thioU supported a level and pattern of cross-linking comparable with that of ES2. Nevertheless, as expected, an excess of the homologous competitor inhibited all cross-linking by thiolated D33, whereas the same (20–40-fold) or greater (200-fold) excess of competitor had no effect on the cross-linking interactions at ES2 and ES1 (Fig. 9 and data not shown). Overall, these observations suggest that the cross-linking subunits of editing complexes can make transient nonspecific contacts with RNA; however, the similar interactions at ES2 and ES1 are significantly more stable.

DISCUSSION

The molecular basis of substrate recognition by editing complexes and the regulation of RNA editing in the single mitochondrion of trypanosomes are still poorly understood. The purpose of these studies was to dissect functional substrate determinants proximal to a site for full-round U insertion catalyzed by purified editing complexes.

Combined, these observations and our previous study (41) have important implications on the mechanisms of substrate recognition by editing complexes. First, the overall recognition of the insertion substrate outside the internal loop is sequence-independent. This notion is consistent with structural probing studies suggesting that related secondary structures of different mRNA/gRNA pairs may be important for editosome recognition (53). Our analysis of a minimal A6 RNA pair for full-round insertion at ES2 (41) showed that the sequence and base composition of the parental helices flanking the editing site, including the pre-mRNA purine-richness (93%) in the left duplex, are not required for efficient editing. We had recently shown that the all-purine pre-mRNA protrusion could be replaced by a pyrimidine-rich stretch (41). Although natural 5’ polypurine runs in the A6 pre-mRNA are dispensable for the basic insertion reaction in vitro, it is conceivable that these structures are specifically recognized in vivo by factors to promote nucleation with the complementary uridylate tail of gRNAs or stabilization of the duplex. In line with this notion, an editing complex subunit (KREPA4) was recently reported to exhibit binding specificity for a gRNA 3’ U-tail (37). Furthermore, a proposed accessory factor (REAP-1) preferentially binds to purine-rich transcripts such as pre-mRNAs (60).

Notably, substrates with completely artificial 11-bp duplexes
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(i.e. one helical turn of RNA) flanking ES2 support efficient insertion (e.g. Pair-11 and Pair-12; Fig. 2). We speculate that one or both duplexes flanking ES2 is recognized by KREN2, an endonuclease subunit that specifically serves in insertion (14), and bears an RNase III-like domain and one double-stranded RNA binding motif (1, 8). Interestingly, the 11-bp artificial helices in our substrates may be minimal in length, as structural studies of highly conserved double-stranded RNA binding motifs in other systems indicate that these proteins typically interact with 16-bp (~1.5 helical turns of dsRNA; Ref. 61). Moreover, a recent study proposed that the smallest dsRNA substrate for the single double-stranded RNA binding motif in a bacterial RNases III is 11 base pairs (62). KREN2 may dimerize (24) like other class 1 RNase III enzymes (63), so that each double-stranded RNA binding motif could contact one of the 11-bp helices flanking ES2 in our constructs.

Typical double-stranded RNA binding motifs specifically bind the A-form of dsRNA through interactions that are adapted to the shape of the helix, are sequence-independent, and primarily involve hydrogen bonds with ribose 2'-hydroxyls (64). This is consistent with the observed induction of both U insertion and editing complex photo-cross-linking interactions at ES2 by the presence of (B-form) dsDNA in the flanking helices (Fig. 4B). Also in line with this notion, 2' deoxy substitutions in one strand caused more moderate negative effects (Figs. 4, B and E) possibly because DNA/RNA heteroduplexes retain half of the hydroxyls and conserve more the RNA than the DNA helical shape (65).

An 11-bp DNA duplex 5 bp downstream of ES2 (Pair-25) markedly reduced accurate cleavage and, instead, stimulated low level cryptic pre-mRNA cuts near ES2 (Fig. 4C). Such an effect on scissile-bond selection suggests that the modified riboses in the right duplex help position KREN2 to precisely cleave the bond at the single-/double-strand junction (ES2). Interestingly, the significant cleavage inhibition correlates with a dramatic reduction of editing complex cross-linking at the same site (Fig. 4E). Similarly, the presence of helical DNA 4 bp upstream the internal loop moderately inhibited both cleavage and RNA-protein interactions at ES2.

Together, these data suggest that the editing complex makes important contacts with both helices flanking ES2. Such contacts with the downstream anchor duplex appear particularly relevant for efficient and accurate pre-mRNA cleavage as well as for cross-linking interactions at the editing site. 2'-Hydroxyl groups in the pre-mRNA protrusion (i.e. Pair-13; Fig. 3A) also contribute to the insertion reaction but more moderately. However, a construct with the combined modifications of Pairs 13 and 14 significantly inhibited cleavage (data not shown) and, thus, further emphasize the impact of the upstream pre-mRNA structure on this editing step. Single ribose 2' substitutions at ES2 including 2'-H, 2' -OCH3, and 2'-F showed that the 2'-hydroxyl at this site is critical for either pre-mRNA cleavage or prior ES2 recognition. Interestingly, ES2 with either a 2'-OH or 2'-H supported the same pattern of editing complex cross-linking interactions at the editing site. Because protein-RNA photo-cross-linking is sensitive to substrate conformational changes, we suspect that most protein interactions involved in ES2 recognition are unaffected at the 2'-H modified site. A potential role of the 2'-hydroxyl at ES2 in catalysis could involve 1) formation of a critical hydrogen bond with the KREN2 endonuclease, 2) coordination of a divalent cation cofactor, or 3) direct nucleophilic attack on the scissile bond. Editing complex-catalyzed cleavage of pre-mRNA containing a single 32P at ES2 produces a 5'-labeled downstream fragment (data not shown) rather than a 3'-labeled upstream fragment as would be expected if the 2'-OH group forms a 2',3' cyclic phosphate upon cleavage, as occurs with RNase A (39). This is consistent with previous RNase mapping that deduced the 5'P and 3'OH nature of the termini produced at ES2 (45, 46) and also is in line with an RNase III Mg2+-dependent type of processing (45, 46). Further work will be needed to dissect the precise role/s of the 2'-hydroxyl group at ES2 in pre-mRNA cleavage and/or editing site recognition.

Our deoxy substitutions within the ES2 internal loop were also quite informative. Interestingly, substitution of the three residues 5' of the guanosine (bearing a critical 2'-OH) at ES2 affected neither cleavage nor U insertion; however, substitution of all apposing loop gRNA residues strongly inhibited both insertion at pre-mRNA cleavage and cross-linking activities at ES2. These results were unanticipated as they reveal that the gRNA strand in the internal loop is an important determinant of full-round U insertion at the level of pre-mRNA cleavage and suggest that the proximal pre-mRNA 2'-OH groups upstream of the guanosine at ES2 are less relevant for substrate cleavage. Because the relative level of insertion and cleavage were similar, these 2' deoxy modifications appear to have little or no effect on either U addition (by the terminal U-transferase, KRET2) or RNA ligation (by KREL2) in the insertion cycle. It is intriguing that the combined pre-mRNA/gRNA substitutions in the loop were more inhibitory (in all assays tested) than gRNA modifications alone because pre-mRNA substitutions had no effect. It is possible that the pre-mRNA deoxynucleotides facilitate a conformation of the substituted gRNA loop that is particularly inhibitory.

Several editing complex subunits contain conserved motifs that may bind single-stranded RNA around an ES. For example, KREPA1 (also termed band II, LC-1; Refs. 1, 5, and 6) has an oligonucleotide/oligosaccharide binding (OB)-fold that could bind single-stranded RNA in interactions independent of ribose 2'-hydroxyls (66). KREPA1 was speculated to have a role in recognition of the editing substrate and possible coordination of an insertion cycle (1). Consistent with this concept, we recently proposed that the related OB-fold containing KREPA2 (band III, LC-4) directly binds a site for full-round U deletion (Ref. 35). Additional related subunits of the editing complex were also proposed to conserve an OB-fold. Three subunits including KREPA1 and KREPA2 also bear C2H2 zinc finger domains that could potentially bind single-stranded RNA (64, 67). Furthermore, the RNase III-like insertion endonuclease KREN2 is expected to cleave single-stranded RNA at a single-/double-strand junction, unlike typical RNase III enzymes that cleave dsRNA. KREN2 has one double-stranded RNA binding motif, one RNase III domain, and also a U1-like zinc finger (1). Perhaps one or more of these motifs specifically interact with internal loop determinants involved in scissile-bond selection at insertion sites.
All ribose 2′-deoxy substitutions tested in this study are summarized in Fig. 6. Notably, proximal changes that significantly decreased pre-mRNA cleavage were also associated with a parallel inhibition of editing complex cross-linking at ES2. Thus, the cross-linking assay we introduced here not only revealed for the first time direct editing complex interactions at an ES for full-round insertion but also can help define ribose 2′-OH groups in cis (pre-mRNA) or in trans (gRNA) that affect the efficiency of both cleavage catalysis and photo-cross-linking at sites targeted by editing complexes. Work is in progress to identify the photo-cross-linking subunits; however, due to the similar size and gel mobility of several subunits, identification of the cross-linking proteins is not straightforward. We are currently combining the use of mass spectroscopy techniques and epitope-tagging to produce confirmatory gel-shifts of candidate subunits.

Interestingly, the cross-linking pattern at the insertion ES2 and deletion ES1 (35) in A6 model substrates are similar. Whether or not they play a role in the distinction of editing sites is uncertain, although the cross-linking efficiency at ES1 is significantly greater than at ES2. Notably, the former is the most efficient model ES known for in vitro editing (42). Our competition analyses showed a similar response of both cross-linking and pre-mRNA cleavage activities and suggest a preferential association of editing complexes with the A6 substrate. These results also implied a functional relevance of the cross-linking interactions.

Surprisingly, a transcript (D33) that does not interfere with cross-linking at an ~200-fold excess (35) cross-linked in its thiolated version with editing complexes. However, as expected, these cross-links were very sensitive to low concentrations of unlabeled D33 (competitor), whereas the similar ES2 and ES1 cross-links were resistant to the same or greater concentrations of D33. This suggests that most if not all cross-linking subunits can make transient non-specific contacts with RNA, which may be detected by the sensitive 4-thioU photo-reactant. In contrast, the associations at ES2 and ES1 are relatively stable. These stabilized interactions, however, are sequence-independent as they are conserved at both ESs tested and may reflect recognition of helical irregularities (e.g. potential ESs) in hybrid substrates. Our laboratory is currently exploring these possibilities.

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