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In vitro* RNA editing-like activity in a mitochondrial extract from *Leishmania tarentolae

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Communicated by J.H.Miller

A mitochondrial extract from *Leishmania tarentolae* directs the incorporation of uridylylate (U) residues within the pre-edited domain of synthetic cytochrome *b* (CYb) and NADH dehydrogenase subunit 7 mRNA. This has several characteristics of an *in vitro* RNA editing activity, but no direct evidence for involvement of guide RNAs was obtained. Inhibition by micrococcal nuclease suggests a requirement for some type of endogenous RNA. The limitation of internal U-incorporation to the pre-edited region in the CYb mRNA and the inhibition by deletion or substitution of both mRNA anchor sequences for CYb gRNA-I and -II could be consistent either with a gRNA-mediated process or a secondary structure-mediated process. A low level of incorporation of [α -³²P]CTP occurs at the same sites as UTP. Internal U-incorporation activity is selectively inhibited by heterologous RNAs, suggesting an involvement of low affinity RNA-binding proteins which can be competed by the added RNA.

Key words: *Leishmania*/mitochondria/RNA editing/TUTase

Introduction

RNA editing of mitochondrial transcripts in trypanosomatid protozoa involves the addition and occasional deletion of uridine (U) residues within coding regions and in some cases within 3' and 5' untranslated regions. The extent of editing ranges from a few nucleotides at a few sites to hundreds of nucleotides at hundreds of sites. Editing occurs post-transcriptionally in an overall 3' to 5' direction, and is mediated by small complementary guide RNAs (gRNAs) which contain the edited sequence information (for recent reviews see Hajduk *et al.*, 1993; Simpson *et al.*, 1993; Sloof and Benne, 1993; Stuart, 1993).

Two basic models for the mechanism of editing have been proposed: (i) the enzyme cascade model (Blum *et al.*, 1990; Decker and Sollner-Webb, 1990; Harris and Hajduk, 1992), which invokes a type of mismatch repair involving a mitochondrial endonuclease, a terminal uridylyl transferase (TUTase) and an RNA ligase and (ii) the transesterification model (Blum *et al.*, 1991; Cech, 1991),

which invokes a transfer of U residues from the 3' oligo(U) tail of the gRNA to the mRNA by two successive transesterification reactions. A hybrid model involving the transfer of U residues from the gRNA to the editing site by a cleavage-ligation mechanism has also been proposed (Sollner-Webb, 1992). The main evidence for the transesterification model is the existence of the predicted gRNA/mRNA chimeric molecules in steady state RNA (Blum *et al.*, 1991; Koslowsky *et al.*, 1991; Read *et al.*, 1992). Chimeric molecules can be formed *in vitro* from synthetic gRNAs and synthetic pre-edited mRNAs in the presence of mitochondrial extracts from trypanosomatids (Blum and Simpson, 1992; Harris *et al.*, 1992; Koslowsky *et al.*, 1992). This *in vitro* chimera-forming activity is dependent on the presence of an anchor duplex between the gRNA and mRNA (Blum and Simpson, 1992). The mechanism of chimera formation *in vitro* is still an open question, as is the participation of chimeras as true editing intermediates *in vivo*.

Several classes of ribonucleoprotein (RNP) complexes involving gRNAs have been detected in mitochondrial extracts from trypanosomatids. Pollard *et al.* (1992) reported that in *Trypanosoma brucei*, two RNP complexes can be detected: a 19S complex which contains gRNA, TUTase, RNA ligase and chimera-forming activity, and a 35-40S complex which has in addition pre-edited RNA, but lacks tightly bound TUTase. Göringer *et al.* (1994) and Read *et al.* (1994) have identified by native gel electrophoresis several mitochondrial RNP complexes from *T.brucei* which interact with exogenous synthetic gRNAs and mRNAs. Peris *et al.* (1994) have described two classes of RNP complexes in a mitochondrial extract from *Leishmania tarentolae*: (i) T-complexes, which contain gRNAs and mRNAs that are 3' end-labeled with [α -³²P]UTP by an endogenous TUTase activity, and which sediment at 10S in glycerol and migrate in native gels as ~6 bands; and (ii) G-complexes, which contain gRNAs and sediment at 25S in glycerol, and exhibit an *in vitro* RNA editing-like activity. This RNA editing-like activity which involves the incorporation of U residues into the pre-edited region of a synthetic mRNA is described in detail in this paper. The availability of this system should aid in the elucidation of the mechanism of RNA editing.

Results

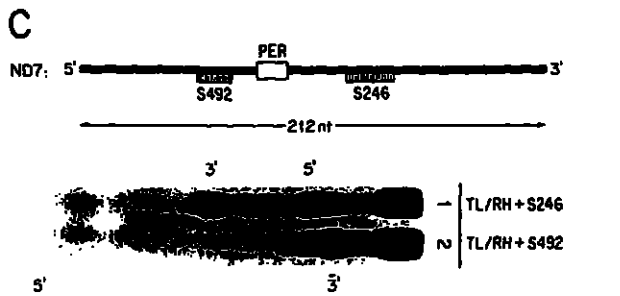
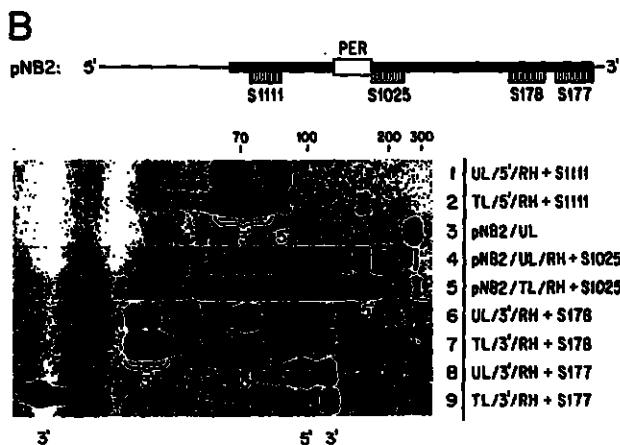
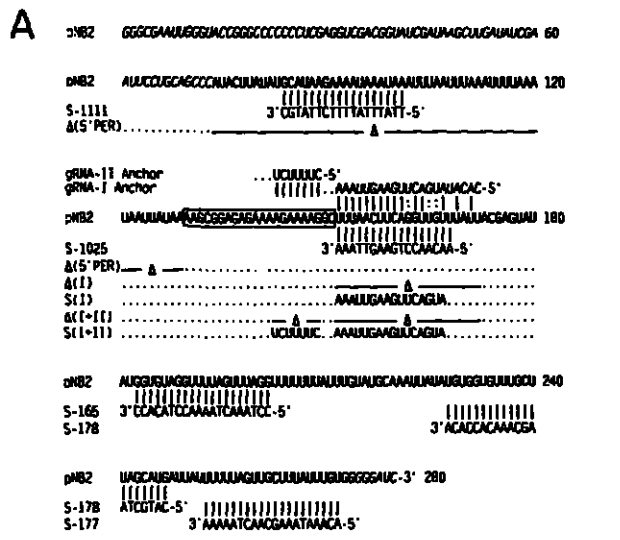
U residues are incorporated internally in a synthetic pre-edited cytochrome b mRNA in the presence of mitochondrial extract

In vitro synthesized pNB2 RNA [which contains the 5' portion of the cytochrome *b* (CYb) mRNA, Simpson *et al.*, 1992; Figure 1A] was labeled with [α -³²P]UTP in the presence of the Triton lysate (TL) mitochondrial extract, which consists of a kinetoplast mitochondrial fraction

homogenized in 0.3% Triton X-100. Incorporation of [α - 32 P]UTP during incubation with TL extract occurred both at the 3' terminus of the RNA, as expected from the known 3' TUTase activity present in the mitochondrial extract (Bakalara *et al.*, 1989), and internally. The approximate localization of the incorporated U residues within the pNB2 molecule after incubation with mitochondrial extract was determined by RNase H digestion after hybridization with specific oligonucleotides (Donis-Keller, 1979; Figure 1B). pNB2 RNA which was uniformly labeled (UL) during T7 transcription was used as a control substrate. The relative extent of internal incorporation of U residues into pNB2 RNA under optimal conditions varied from 20 to 60%, depending on the mitochondrial preparation. The relative and absolute internal incorporation of U residues was also affected by the amount of

mitochondrial extract used in the reaction and was titrated for each mitochondrial preparation. The approximate absolute efficiency of the internal U-incorporation was calculated to be in the order of 1 in 1000 molecules, assuming an average of 5–10 U residues incorporated per molecule (see below).

Internal incorporation of labeled U residues was localized between the positions of the S1111 and S1025 oligonucleotides by the experiments in Figure 1B. As is apparent in lane 2, ~99% of internal U-incorporation occurs downstream of S1111. In lanes 6–9, the 3' fragment released by digestion with S1025 and RNase H was gel-isolated and subjected to additional RNase H digestions. The absence of significant incorporation between S1025 and S178 or S177 is shown in lanes 7 and 9. These data localize internal U-incorporation to a 60 nucleotide region encompassing the 22 nucleotide pre-edited region.



Nearest neighbour analysis of U residues incorporated internally

pNB2 RNA was labeled with [α - 32 P]UTP in the presence of TL extract, and the gel-purified labeled RNA was digested with the S165 oligomer and RNase H. The 5' fragment was isolated and digested with RNase T2, and the 3' monophosphates separated by 2-D TLC (Figure 2A). This yielded 51% Up, 33% Gp, 15% Ap and 1% Cp, values which are inconsistent with complete precise editing, which would be predicted to yield 62% Up, 15% Gp, 20% Ap and 3% Cp (Feagin *et al.*, 1988), or with completely random insertion of U residues (29% Up, 20% Gp, 35% Ap and 16% Cp). These results, however, confirm that the U residues were incorporated into internucleotide linkages within the 5' RNase H fragment.

Nuclease P1 digestion of the 5' fragment of pNB2 RNA labeled with [α - 32 P]UTP in the presence of TL extract and separation of the mononucleotides by TLC showed that all of the label was in the UMP residue (Figure 2B). No indication of any conversion of the added [α - 32 P]UTP into another nucleotide was obtained.

Fingerprinting of [α - 32 P]UTP-labeled pNB2 RNA by RNase T1, A and CL3 identifies the pre-edited region as the area of internal U-incorporation

The 5' RNase H/S1025 fragment of pNB2 RNA which was labeled with [α - 32 P]UTP in the presence of TL was

Fig. 1. U-incorporation into synthetic pNB2 and ND7 RNAs.

(A) *Leishmania tarentolae* CYb-derived sequence is indicated in bold, Bluescript vector-derived sequence appears italicized. The pre-edited region is boxed. Identical residues are indicated as dots, deletions appear as Δ . The 5' and 3' ends of all mutants (Δ (5'PER), Δ (I), Δ (I+II), S(I), S(I+II)) are identical to pNB2 RNA. The positions of the antisense oligomers S1111, S1025, S165, S178 and S177 are indicated. Only the anchor-forming portions of CYb gRNAs I and II (Blum and Simpson, 1990) are shown. Watson-Crick base pairs are depicted by vertical lines. GU base pairs by colons. (B) pNB2 RNA uniformly labeled (UL) or labeled by incubation with TL was analyzed by the RNase H (RH) assay in conjunction with oligomer S1025 (lanes 4 and 5). The 5' portions and the 3' portions were gel-isolated and redigested with RNase H and oligomer S1111 (lanes 1 and 2), and S178 (lanes 6 and 7) or S177 (lanes 8 and 9), respectively. The positions of ethidium-stained RNA size markers are indicated. In the diagram, the thin portion of the horizontal line represents vector-derived sequence, and the thick central portion represents *L.tarentolae* CYb sequence. PER = pre-edited region. (C) ND7 mRNA was incubated with TL and subsequently analyzed by the RNase H assay in conjunction with oligomer S246 (lane 1) or S492 (lane 2).

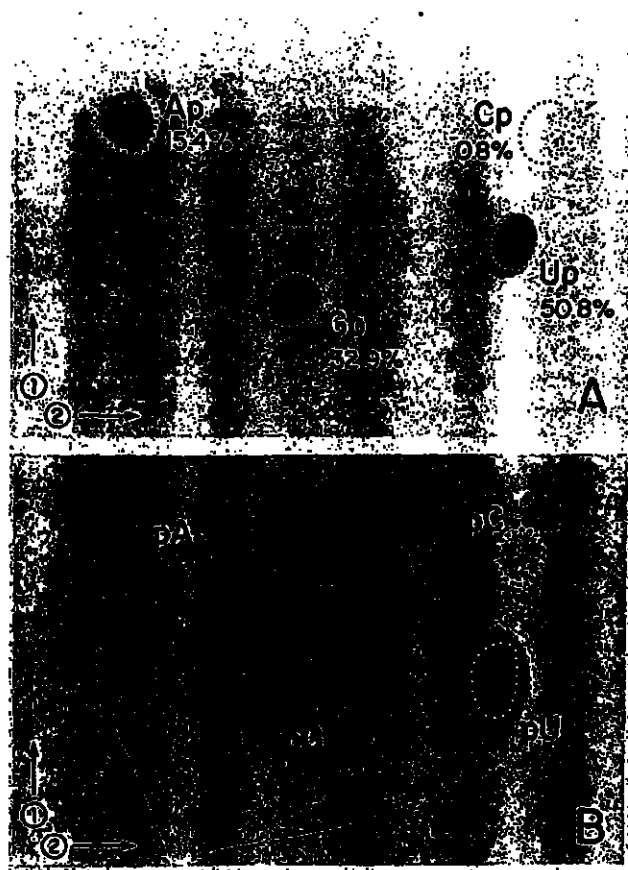


Fig. 2. Nearest neighbour and nucleotide analyses of internally incorporated [α - 32 P]UTP. (A and B) pNB2 RNA labeled with [α - 32 P]UTP in the presence of TL extract was digested with RNase H and the oligomer S165. The 5' fragment was gel-isolated, digested with RNase T2 (A) or nuclease P1 (B) and the resulting 3' monophosphates (A) or 5' monophosphates (B) separated by 2-D TLC. The relative amounts of label in each of the 3' monophosphate spots are indicated.

digested with RNase T1, A or CL3, and the cleavage products analyzed by gel electrophoresis (Figure 3). In each case, control transcripts uniformly labeled with [α - 32 P]ATP, GTP, CTP or UTP were digested in order to identify specific fragments. All three nucleases release fragments containing 3' phosphates.

The results of digestion with RNase T1, which cuts 3' of G residues, are shown in Figure 3A, lanes 3 and 4. Only 2.5% of the total incorporated label appears within the largest fragment, which contains the sequence upstream of the pre-edited region as well as the last three editing sites of editing block II (Figure 4A). This result, in conjunction with the RNase H results presented above, suggests that >95% of the internally incorporated U residues are localized within the pre-edited region. In addition, the fact that the large fragment migrated predominantly as a 43mer suggests that the U-incorporation in the 42mer fragment which did occur was limited to a single U insertion, which could in fact be at one of the three editing sites in this region. The absence of significant U-incorporation downstream of the pre-edited region is indicated by the above RNase H results in Figure 1B and by the fact that the S1025 oligonucleotide could hybridize to allow digestion with RNase H. Furthermore, if U-incorporation occurred within the stretch of U residues

immediately downstream of the pre-edited region, this would give rise to T1 digestion fragments longer than 12 nucleotides in length, and fragments of this size in Figure 3A contain <2% of the total label incorporated. The majority of the label after RNase T1 digestion appears in fragments 5–10 nucleotides in size, as well as in the guanosine-3' monophosphate (Gp) spot. The Gp spot contains ~13% of the total label incorporated, a fact which identifies editing site 2 (block I) and/or editing site 6 (block II) as frequent sites of *in vitro* U-incorporation. Fragments six nucleotides and above in size appear as a uniform ladder, indicating that these fragments are probably created by insertion of increasing numbers of U residues into the same fragment, most likely into the two AAAAG pentamers which together contain six of the 15 *in vivo* editing sites (Figure 4A).

The results obtained after digestion with RNase A, which cuts 3' of C and U residues, are presented in Figure 3B. The labeled 18mer present in lane 2 must originate from molecules containing a U insertion(s) at editing site 1 or 2 (block I; Figure 4B). As is evident from the RNase T2 nearest neighbour analysis presented above, U residues are not incorporated abundantly into editing site 1, since only 1% of all U residues are incorporated 3' of a C. The next band visible in lane 2 of Figure 3B most likely represents a 17mer and must originate from molecules containing either a U insertion(s) at editing site 3 (block I), or at site 7 (block II) and concomitantly at sites 1 or 2 (block I). All other fragment sizes can be accounted for by various combinations of single or multiple U insertions within the cytosine-bordered 18mer fragment (Figure 4B). The small amount of label present in the Up/Cp spot does not necessarily mean that insertions of more than two U residues per site rarely occur, since the unexpectedly low intensity of the Up/Cp spots in the [α - 32 P]UTP and [α - 32 P]ATP uniformly labeled controls (Figure 3B, lanes 7 and 4, respectively) indicates that stretches of oligo(U) are not well cleaved into single U residues under the digestion conditions employed. Insertion of more than one U per site must actually occur frequently since the RNase T2 nearest neighbour analysis in Figure 2A revealed that half of all incorporated U residues are 3' of other U residues.

The results of digestion with RNase CL3, which cuts 3' of C residues and, at a lower rate, 3' of A and G residues, but does not cleave at U residues, are shown in Figure 3C. Under the digestion conditions employed, the two largest predicted fragments, an 18mer (same fragment as in RNase A digest in Figure 4B) and a 48mer (see sequence in Figure 4B), are the two predominant fragments (Figure 3C, lanes 4–7), although the background of spurious bands is considerably higher than for the RNase T1 (Figure 3A, lanes 6–9) and RNase A (Figure 3B, lanes 4–7) digests. Only ~2% of the total incorporated label appears to be within the 48mer fragment (Figure 3C, lane 2, marked by an arrowhead), which includes the sequence upstream of the pre-edited region as well as the last three editing sites of editing block II. This observation is consistent with the RNase T1 results. Furthermore, the RNase CL3 data provide additional evidence that almost all of the U-incorporation occurs within the 18mer fragment which contains most of the pre-edited region. The number of U residues inserted appears to vary between 1 and ~20.

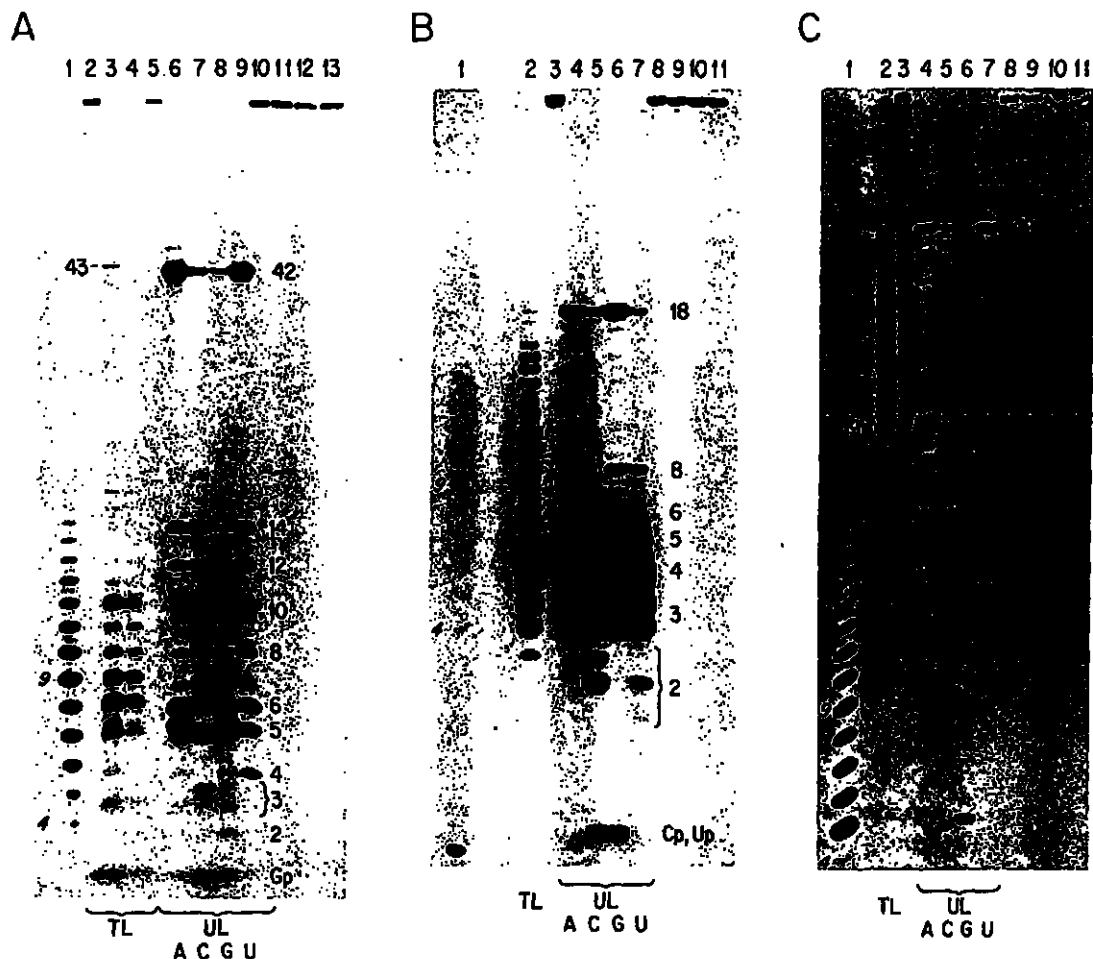


Fig. 3. Fingerprinting analyses of pNB2 RNA containing internal U-incorporations. (A,B,C) pNB2 RNA was either uniformly labeled (UL) with [α - 32 P]ATP, [α - 32 P]CTP, [α - 32 P]GTP or [α - 32 P]UTP to obtain control substrates, or labeled by incubation with TL extract in the presence of [α - 32 P]UTP. The 5' portions were recovered after cleavage with RNase H in conjunction with the oligomer S1025, and used for nuclease digestion and subsequent PAGE analysis. Sizes of predicted RNA oligomers appear on the right, and the positions of oligo(dT) 4mers and 9mers are indicated italicized on the left of each panel. (A) Digestion with RNase T1. Two different TL extracts were used for this experiment and the data obtained were qualitatively equivalent (lanes 3 and 4). Lane 1, oligo (dT)₄₋₂₂ ladder; lanes 2 and 5, TL-incubated RNA, 0 min time points of digests shown in lanes 3 and 4; lanes 6-9, UL RNA, labeled as indicated with A, C, G or U, digested with RNase T1; lanes 10-13, 0 min time points of control digests shown in lanes 6-9. (B) RNase A digestion. Lane 1, (dT)₄₋₂₂; lane 2, TL-incubated RNA digested with RNase A; lane 3, 0 min time point of TL-incubated RNA shown in lane 2; lanes 4-7, UL controls, labeled as indicated, digested with RNase A; lanes 8-11, 0 min time points of digests shown in lanes 4-7. (C) Digestion with RNase CL3. Lane 1, (dT)₄₋₂₂; lane 2, TL-incubated RNA digested with RNase CL3; lane 3, 0 min time point of TL-incubated RNA shown in lane 2; lanes 4-7, UL RNA, labeled as indicated, digested with RNase CL3; lanes 8-11, 0 min time points of digests shown in lanes 4-7.

with the insertion of only one U being somewhat preferred, as shown by the fact that 10-15% of the total label is in the 19mer band. A similar distribution pattern was observed by comparing, on a high resolution 6% polyacrylamide gel, a 5' RNase H fragment derived from pNB2 RNA which was incubated with TL with the same fragment derived from uniformly labeled RNA (data not shown).

Internal U-incorporation into pre-edited ND7 mRNA

Internal U-incorporation into a synthetic pre-edited NADH dehydrogenase subunit 7 (ND7) mRNA was also observed, as shown in Figure 1C. The relative extent of internal incorporation versus the 3' terminal addition was approximately the same as in the case of the pNB2 RNA. The precise localization of the internally incorporated U residues was not determined, but the 5' fragment released by RNase H digestion includes the pre-edited region.

Internal U-incorporation does not occur with several non-edited RNAs

Incubation with the mitochondrial extract of several T7 RNA polymerase-synthesized RNAs which are not edited *in vivo*, including portions of the mitochondrial 9S rRNA, NADH dehydrogenase subunit 4 (ND4) mRNA, cytochrome oxidase subunit I (COI) mRNA and ND1 mRNA, led to a 3' terminal addition of U residues but no apparent internal incorporation (data not shown). ND5 mRNA, however, which is normally not edited, did show a low level of internal incorporation of U residues (data not shown), but this phenomenon was not studied further.

Nucleotide requirements of the internal U-incorporation activity

Internal U-incorporation activity with pNB2 RNA was strongly stimulated by ATP or ADP, somewhat less by

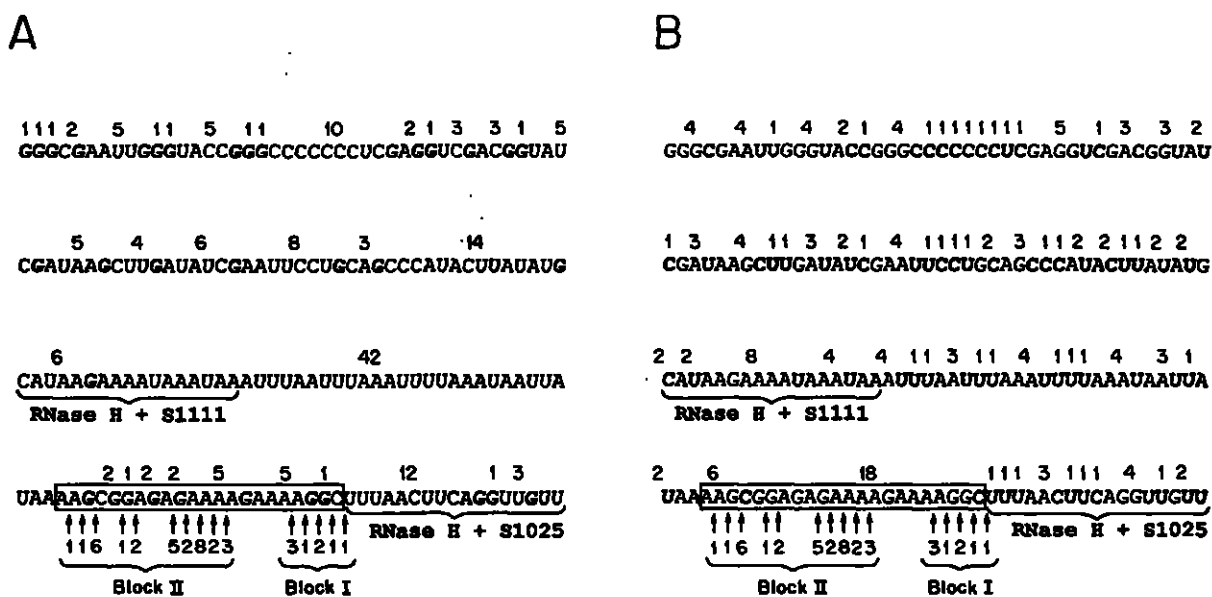


Fig. 4. Predicted fragment sizes of pNB2 RNA digested with RNase T1 or RNase A. (A and B) The sequence of the pNB2 RNA 5' portion used for the fingerprinting analyses in Figure 3 is indicated. The predicted digestion fragment sizes appear above the sequence, flanked by emboldened G nucleotides (A), or emboldened C and U nucleotides (B), 3' of which RNase T1 or RNase A, respectively, are expected to cleave. The localizations of the DNA oligonucleotides S1111 and S1025 used for RNase H cleavage, as well as the sites and numbers of U additions in mature edited CYb mRNA are shown. Block I and II within the boxed pre-edited region refer to the two editing blocks.

AMP or GTP, but not by adenosine or CTP (Figure 5). Optimal internal U-incorporation activity was obtained using ATP plus GTP (Figure 5B). Two analogues of ATP which are non-hydrolyzable or weakly hydrolyzable at the β - γ bond (AMP-PNP and ATP- γ -S) could substitute for ATP in the stimulation of internal U-incorporation activity, but AMP-PCP was not effective (Figure 5A). AMP-PCP, which is non-hydrolyzable at the α - β bond, also could not substitute for ATP in stimulating this reaction (Figure 5A).

Removal of mRNA anchor sequences selectively inhibits internal U-incorporation

The anchor sequences of the substrate pNB2 RNA were modified by *in vitro* mutagenesis. As shown in Figure 1A, each of the two anchor sequences in pNB2 RNA was either deleted [mutants $\Delta(I)$, $\Delta(I+II)$] or substituted with non-base-pairing nucleotides [mutants S(I), S(I+II)]. The results shown in Figure 6A indicate that removal or substitution of the first anchor actually stimulates the internal U-incorporation somewhat, but removal or substitution of both anchors decreases the internal U-incorporation by 55–80%. It should be noted that removal of anchor I does not affect the second anchor, which would still be available for hybridization with gRNA-II.

The residual amount of internal U-incorporation in the double anchor deleted/substituted mutant substrates was also sensitive to removal of ATP from the reaction medium and to micrococcal nuclease digestion (data not shown), suggesting that this residual incorporation occurred by the same mechanism as in the case of the wild-type pNB2 RNA.

Deletion of the 5' flanking sequence of the substrate mRNA [mutant $\Delta(5'PER)$, Figure 1A] almost completely eliminated the internal U-incorporation, without apparent effect on the 3' addition (Figure 6A, lane 8). This could

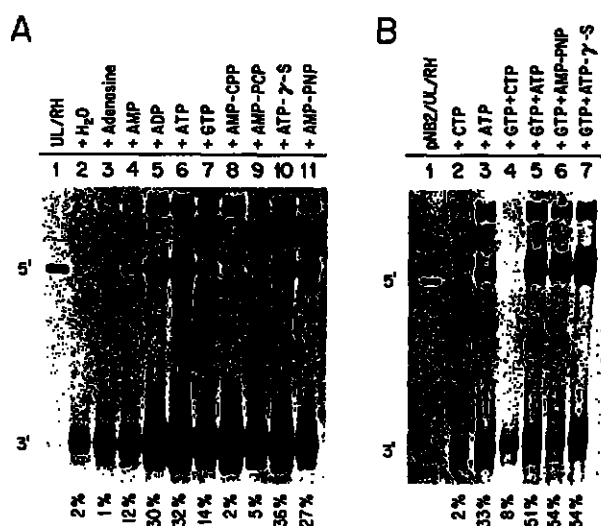


Fig. 5. Nucleotide requirements of internal U-incorporation activity. (A and B) pNB2 RNA uniformly labeled (lane 1) or labeled by incubation with TL (lanes 2–11) was analyzed by the RNase H assay in conjunction with oligomer S165. Incubation with TL was performed under standard conditions, except that only the indicated cold triphosphates were present at 1 mM each. The relative percentages of label incorporation (5' internal versus 3' addition) as well as the positions of the 5' and 3' fragments are indicated.

represent either an effect on secondary structure of the substrate RNA or removal of a *cis*-acting regulatory signal.

A test for the transfer of 3' terminal U residues from [α -³²P]UTP end-labeled CYb-I and CYb-II synthetic gRNAs to pNB2 RNA proved negative (data not shown). However, this is not unexpected, since endogenous gRNAs are present in the TS extract as components of RNP complexes (Peris et al., 1994), and we show below that addition of exogenous synthetic gRNAs to the extract actually inhibits internal U-incorporation activity.

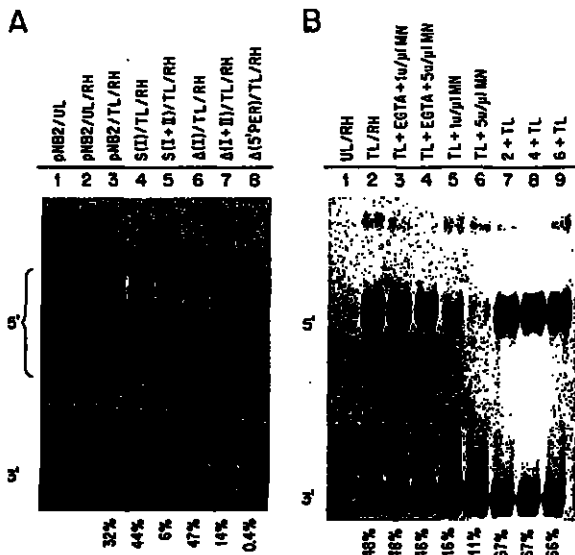


Fig. 6. Removal/modification of CYb mRNA anchor sequences, and micrococcal nuclease digestion. (A) pNB2 RNA or RNA from each of the five mutants was labeled with [α - 32 P]UTP by incubation with TL and subsequently analyzed by the RNase H assay using oligomer S165 (lanes 3–8). The relative percentages of label incorporation (5' internal versus 3' addition) are indicated. Uniformly labeled pNB2 RNA was used as a control (lanes 1 and 2). (B) TL extract was incubated with active micrococcal nuclease (MN) (lanes 5 and 6), or EGTA-inactivated MN (lanes 3 and 4). After EGTA-inactivation of the nuclease in all samples, Δ (I) RNA was added and labeled with [α - 32 P]UTP under standard incubation conditions. Δ (I) RNA labeled by incubation with untreated TL extract served as a positive control (lane 2). Samples pretreated as those shown in lanes 2, 4 or 6 were incubated with an equal amount of fresh TL extract (lanes 7–9). All reactions were subsequently analyzed by the RNase H assay in conjunction with oligomer S165. The micrococcal nuclease concentrations are indicated, as well as the relative percentages of label incorporation (5' internal versus 3' addition). RNase H-digested uniformly labeled Δ (I) RNA was run as a size marker (lane 1).

Internal U-incorporation is selectively inhibited by digestion with micrococcal nuclease

Predigestion of mitochondrial extract with increasing amounts of micrococcal nuclease produced a specific decrease in the extent of internal U-incorporation as compared with 3' terminal U-addition (Figure 6B). EGTA-inactivated nuclease was used as a control for substrate masking (Wang and Gegenheimer, 1990). Addition of nuclease-pretreated extract to an equal amount of untreated extract did not result in inhibition of the internal U-incorporation activity (Figure 6B, compare lane 9 with lane 7). This result suggests that micrococcal nuclease pretreatment does not generate *trans*-acting inhibitors of the activity. These experiments provide evidence for the existence of an endogenous RNA component which is required for the internal U-incorporation activity.

Pretreatment of mitochondrial extract with greater than ~ 10 μ g/ml proteinase K for 30 min at 27°C eliminated both the internal as well as the 3' U-incorporation (data not shown).

C residues are also incorporated internally but less efficiently than U residues

Incubation of pNB2 RNA with [α - 32 P]CTP in the presence of unlabeled ATP and GTP led to internal incorporation of C residues at $\sim 1/20$ the efficiency of [α - 32 P]UTP-

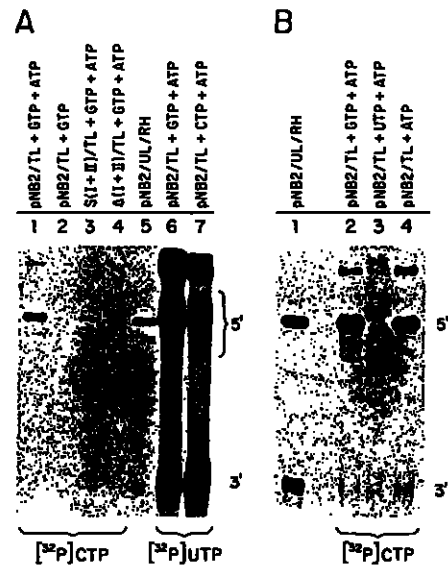


Fig. 7. Internal C-incorporation into pNB2 RNA. (A) pNB2 RNA was labeled using TL and [α - 32 P]CTP (lanes 1 and 2) or [α - 32 P]UTP (lanes 6 and 7). S(I+II) RNA (lane 3) and Δ (I+II) RNA (lane 4) were labeled using TL and [α - 32 P]CTP. Uniformly labeled pNB2 RNA served as a control (lane 5). All reactions were analyzed by the RNase H assay in conjunction with oligomer S165. The various cold triphosphates (1 mM each) added to the reactions are indicated. (B) pNB2 RNA uniformly labeled (lane 1) or labeled by incubation with TL and [α - 32 P]CTP (lanes 2–4) was analyzed by the RNase H assay in conjunction with oligomer S165. The various cold triphosphates (1 mM each) added to the reactions are indicated.

incorporation (Figure 7A). C residues were also added to the 3' end of the substrate RNA, but this occurred at 1/10–1/20 the level of the internal incorporation. This specificity for the internal sites again indicates that the internal nucleotide-incorporation activity involves a different enzymatic machinery than the 3' terminal transferase activity.

Internal C-incorporation activity also requires ATP (Figure 7A), and the C-incorporation is completely inhibited by excess unlabeled UTP (Figure 7B), suggesting that the C residues are incorporated at the same sites as the U residues. This was also indicated by nearest neighbour analysis of RNase T2-digested RNA (data not shown). Furthermore, double anchor deleted/substituted mutant substrate RNAs showed decreased levels of internal C-incorporation (Figure 7A), as was also observed with U-incorporation (see Figure 6A). TLC analysis of mononucleotides released from gel-purified [α - 32 P]CTP-labeled RNA by digestion with nuclease P1 showed that there was no conversion of C to U or any other detectable modification (data not shown).

[α - 32 P]ATP and [α - 32 P]GTP were not incorporated into pNB2 RNA at significant levels (data not shown).

Internal U-incorporation into pNB2 RNA is selectively inhibited by exogenous RNAs

The addition of unlabeled CYb gRNAs I and II (Figure 8A) or ND7 gRNA-II (data not shown) at 1- to 2-fold molar excess over the substrate pNB2 RNA during the incubation with mitochondrial extract decreased the relative extent of internal U-incorporation in pNB2 RNA by $\sim 70\%$, without affecting the absolute level of 3'

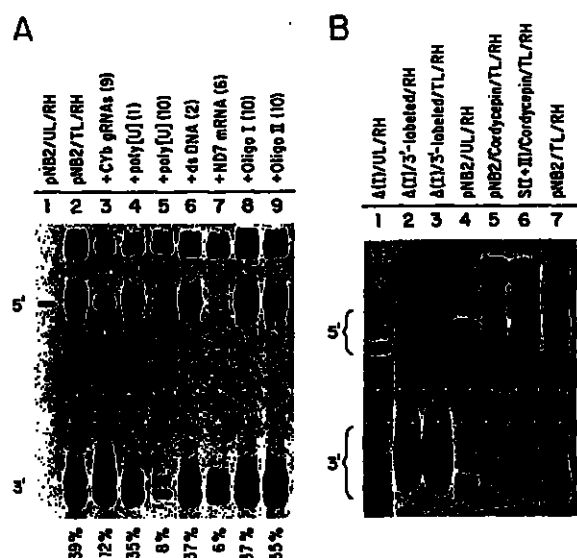


Fig. 8. Specific inhibition of internal U-incorporation by RNA, and lack of U-internalization from the 3' end of pNB2 RNA. (A) pNB2 RNA was labeled with $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ by incubation with TL in the presence of cold synthetic CYb gRNAs I and II (lane 3), poly(U) (lanes 4 and 5), an 87 bp PCR fragment (lane 6), synthetic ND7 mRNA (lane 7), two different oligodeoxynucleotides (lanes 8 and 9) or without addition of heterologous RNA (lane 2). The numbers in parentheses indicate the number of pmol nucleic acids added. Five pmol of pNB2 substrate RNA was present in each reaction. Uniformly labeled pNB2 RNA served as a control (lane 1). All reactions were analyzed by the RNase H assay using oligomer S165. The relative percentages of label incorporation (5' internal versus 3' addition) are indicated. (B) pNB2 RNA or S(I+II) RNA containing cordycepin (3' deoxyadenosine) at the 3' ends (lanes 5 and 6) were labeled with $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ by incubation with TL and subsequently analyzed by the RNase H assay in conjunction with oligomer S165. Gel-isolated, $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ 3' end-labeled $\Delta(I)$ RNA was either directly digested with RNase H (lane 2), or first incubated with TL in the absence of $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ (lane 3). pNB2 RNA without cordycepin served as a control for the activity of the TL extract (lane 7). RNase H-digested uniformly labeled $\Delta(I)$ RNA (lane 1) and pNB2 RNA (lane 4) were run as size markers.

terminal U-addition. ND7 mRNA also showed a selective inhibition of the internal U-incorporation activity (Figure 8A). Poly(U) had a similar effect although there was also an inhibition of the 3' terminal U-addition at high levels of poly(U). Single-stranded DNA (oligonucleotides) present in 2-fold molar excess (Figure 8A) or double-stranded DNA in 3-fold molar excess (data not shown) had no effect. *Escherichia coli* 5S rRNA, rRNA and tRNA showed a specific inhibition of internal U-incorporation at 10-fold higher concentrations (data not shown).

Heparin (5 $\mu\text{g}/\text{ml}$), which has been shown previously (Bakalara *et al.*, 1989) to inhibit 3' non-specific U-addition, also inhibited internal U-incorporation activity (data not shown).

Internal U-incorporation activity does not require the 3' OH of the mRNA substrate

Addition of 3' deoxyadenosine (cordycepin) to the 3' termini of pNB2 or S(I+II) RNA practically eliminated 3' terminal U-addition (Figure 8B), but still allowed internal U-incorporation to occur. This indicates that the internal incorporation activity does not require the 3' hydroxyl of the substrate mRNA.

A direct test for the transfer of labeled U residues from the 3' terminus of the mRNA to intramolecular sites is also shown in Figure 8B. RNA which had been enzymatically 3' end-labeled with $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ using poly(A) polymerase was incubated in the presence of TL extract and then analyzed by the RNase H-oligomer assay. No indication of a transfer of label from the 3' terminus to the internal sites was observed.

Discussion

We have shown the presence of an RNA editing-like activity in a mitochondrial extract from *L.tarentolae* in which U residues are incorporated internally into the pre-edited region of a synthetic mRNA. There is no direct evidence for involvement of endogenous gRNAs in this activity. The precise localization of incorporated U residues into the CYb pre-edited region, which is a sequence defined by two overlapping gRNAs, is consistent either with an involvement of endogenous gRNAs or a requirement for a specific secondary structure. The failure to eliminate editing completely by deletion or substitution of mRNA anchor sequences, and the low levels of internal U-incorporation occurring elsewhere in the pNB2 RNA as well as in the ND5 RNA, could be explained by the intervention of misleading gRNAs, as has been shown to occur *in vivo* (Sturm *et al.*, 1992; Maslov *et al.*, 1994). The loss of activity by removal of the 5' flanking mRNA sequence is consistent either with the secondary structure model or with a requirement for a *cis*-acting signal. The micrococcal nuclease inhibition results indicate a requirement for some endogenous RNA, and this could represent either gRNA or some as yet unidentified RNA component.

Although internal U-incorporation occurs almost entirely within the pre-edited region, the T2 nearest neighbour results indicate that the editing within that region is not precise but is also not completely random. Evidence for editing at several specific *in vivo* sites was obtained by the RNase T1 and RNase A fingerprint data, and the RNase CL3 data showed that one to ~20 U residues were inserted within the pre-edited region. We speculate that this activity may represent an initial non-specific phase of the editing process which then somehow becomes specific due to the involvement of endogenous gRNAs. We speculate that the extract may lack functional yet undefined specificity factors which are involved with editing *in vivo*. These results are also compatible with a model for editing in which U residues are inserted randomly into a defined domain, and in which the role of the gRNA is to 'freeze' the correct pattern and prevent further editing (Decker and Sollner-Webb, 1990).

Internal incorporation of C residues into pNB2 RNA, which occurs at a 20-fold lower efficiency than internal U-incorporation, was not analyzed in detail, but appears to be mediated by the same mechanism, since it is competed by excess unlabeled UTP. A low extent of 3' C addition also occurs, but at a 10- to 20-fold lower rate than internal C-incorporation. This differs from the results obtained using $[\alpha\text{-}^{32}\text{P}]\text{UTP}$, in which there was an approximately equal incorporation 3' and internally. This specificity of C-incorporation for internal sites provides additional evidence that the internal and 3' addition of

nucleotides are directed by different enzymatic mechanisms. It is appealing to speculate that the incorporation of C residues *in vitro* is actually a type of aberrant editing produced by the 'G' guiding nucleotides in the gRNAs which normally guide the insertion of U residues (Blum *et al.*, 1990). Furthermore, it is intriguing, and possibly relevant, that RNA editing of mitochondrial transcripts in another lower eukaryote, *Physarum polycephalum*, involves the incorporation of C residues rather than U residues (Mahendran *et al.*, 1991). It has also been shown that *in vitro* gRNA/mRNA chimera formation can proceed with a 3' terminal C on the gRNA moiety (Harris and Hajduk, 1992).

The specific inhibition of internal U-incorporation by addition of exogenous RNA is possibly interpretable in terms of the presence of low affinity RNA-binding proteins which appear to be required for this activity. Saturation of TS extract with *E. coli* or yeast rRNA has been found to cause a disruption of several low affinity RNA-protein interactions in the T-complexes (E.Byrne, F.Bringaud and L.Simpson, unpublished results), and we speculate that a similar disruption occurs in the G-complexes which affects the internal U-incorporation activity.

This *in vitro* RNA editing-like system may provide a starting point for the analysis of the detailed mechanism of RNA editing of mitochondrial transcripts in kinetoplasts, and for the identification of components of the editing machinery.

Materials and methods

Cell culture, mitochondrial isolation and preparation of mitochondrial extracts

Leishmania tarentolae (UC strain) cells were grown as described previously (Simpson and Braly, 1970) to late log phase ($1-2 \times 10^8$ cells/ml) and used for isolation of the kinetoplast mitochondrial fraction by flotation in Renografin density gradients (Braly *et al.*, 1974; Simpson and Simpson, 1978). The isolated mitochondria were resuspended at a concentration of 1-5 mg protein/ml in 20 mM HEPES (pH 7.5), 100 mM KCl, 0.2 mM EDTA, 10-20% glycerol and frozen at -80°C . TL mitochondrial extract was prepared as described (Simpson *et al.*, 1992). Extracts were either used immediately or stored frozen at -80°C . The internal U-incorporation activity was not greatly affected by freezing and thawing the TL extract several times over a period of several months.

In vitro synthesis of mRNAs and gRNAs

With the exception of the recombinant pBluescript SK(-) plasmid pNB2, all transcription-competent DNA templates were prepared by PCR using 5' primers which contain the T7 promoter sequence. The PCR-amplified DNA fragments were generated by using *L.tarentolae* maxicircle DNA and the following primer sets: S244/S245 (ND7 mRNA), S656/S657 (gCYb-I), S658/S659 (gCYb-II) and S270/S288 (gND7-II). Construction of the pNB2 plasmid, which contains the 5' portion of the *L.tarentolae* *Cyb* gene, has been described previously (Simpson *et al.*, 1992).

In vitro transcription of mRNAs was performed as described previously (Bakalara *et al.*, 1989; Simpson *et al.*, 1992). To obtain uniformly labeled (UL) transcripts, [α - ^{32}P]UTP, [α - ^{32}P]ATP, [α - ^{32}P]CTP or [α - ^{32}P]GTP was included in the transcription reaction. The short gRNA transcripts were produced using conditions described elsewhere (Milligan *et al.*, 1987). All RNA transcripts were gel-isolated from polyacrylamide-urea gels prior to use.

Labeling of RNA in the presence of mitochondrial extract

T7 RNA polymerase-synthesized RNA (0.5-1 μg) was incubated in the presence of TL mitochondrial extract (10-30 μg protein) at 27°C for 40-60 min in a 50 μl reaction volume. Under standard, optimized conditions, the reaction contained 5 mM HEPES (pH 7.5), 60 mM KCl, 3 mM potassium- PO_4 (pH 7.5), 6 mM magnesium-acetate, 20 mM DTT,

2 mM spermidine, 1 mM ATP, 1 mM GTP, 1 μM unlabeled UTP and 25 μCi [α - ^{32}P]UTP (800 Ci/mmol). Note that CTP was not included in the reaction mixture in order to eliminate transcription, which has been shown to require the presence of all four triphosphates (Bakalara *et al.*, 1989). A titration showed that ~ 1 mM ATP was required for maximal stimulation of the internal U-incorporation (data not shown). Spermidine was found to significantly enhance the amount of internal incorporation of U residues and was routinely used in the reaction. In the absence of spermidine, only 2-5% of the relative label incorporation was internal (data not shown).

When assaying for CTP-incorporation, 1 μM unlabeled CTP and 25 μCi [α - ^{32}P]CTP (800 Ci/mmol) were added to the reaction mixture, and UTP was omitted. When assaying for ATP-incorporation, 1 μM unlabeled ATP and 25 μCi [α - ^{32}P]ATP were added to the reaction mixture, UTP was omitted, and unlabeled ATP was omitted or replaced by 1 mM CTP. When assaying for GTP-incorporation, 1 μM unlabeled GTP and 25 μCi [α - ^{32}P]GTP were added to the reaction mixture, UTP was omitted, and unlabeled GTP was omitted or replaced by 1 mM CTP.

Triton X-100 is not required for internal U-incorporation activity, since an extract prepared from frozen-thawed, homogenized mitochondria without detergent was almost as active with pNB2 RNA as the TL (data not shown).

RNase H assay to detect internal incorporation of label

After incubation with mitochondrial extract, the reaction mixture was extracted with phenol-chloroform (1:1) and the RNA separated on a preparative 8% polyacrylamide-7.5 M urea gel. Only limited degradation of the input substrate RNA was observed after incubation with the mitochondrial extract, and the labeled intact input RNA could be visualized as an ethidium-stained band and recovered by elution and subsequent ethanol precipitation. The RNA was hybridized to specific oligonucleotides and digested with RNase H (Donis-Keller, 1979). Each 20 μl reaction mixture contained 0.5-1 μg RNA, 0.1-0.2 μg DNA oligomer, 50 mM Tris-HCl (pH 7.5), 0.1 M KCl, 10 mM MgCl_2 , 0.1 mM DTT, 10 $\mu\text{g/ml}$ BSA and 0.2-0.3 units RNase H (Pharmacia). RNase H digestion was allowed to proceed for 1 h at 37°C . Visualization of incorporated label was achieved by analytical gel electrophoresis (8% polyacrylamide-7.5 M urea gels) and subsequent autoradiography of the dried gel.

RNase T2 nearest neighbour and nuclease P1 analyses

Gel-isolated, labeled RNA fragments were digested with RNase T2 in 50 mM Na-acetate (pH 5.2), 2 mM EDTA at 37°C for several hours. Digested labeled RNA (5 μl) was loaded onto Polygram CEL 300 cellulose plates (Macherey-Nagel) for TLC analysis. RNase T2-digested carrier yeast tRNA (40 μg) was loaded onto each plate in order to allow visualization of the separated 3' monophosphate spots by UV illumination. Separation was achieved by 2-D TLC in isobutyric acid/ $\text{NH}_4\text{OH}/\text{H}_2\text{O}$ (58/4/38) in the first dimension, and saturated $(\text{NH}_4)_2\text{SO}_4$ /1 M Na-acetate (pH 5.2)/isopropanol (80/18/2) in the second dimension. After autoradiography the areas on the TLC plates containing the monophosphates were excised, and the amount of label incorporated was determined by liquid scintillation counting.

Nuclease P1 digestion of gel-isolated, labeled RNA fragments was performed in 50 mM Na-acetate (pH 5.2), 0.4 mM ZnCl_2 at 37°C for several hours. 5 μl digested RNA and 0.5 μl of a mononucleotide solution (50 mM each NMP) were loaded onto each TLC plate, and separation and detection were achieved as described above.

RNA fingerprinting analyses

The reaction conditions were optimized for each enzyme, using as a control the purified 5' fragments of uniformly labeled pNB2 RNA cleaved with RNase H in conjunction with the oligonucleotide S1025.

RNase T1 digestion was performed in a 5 μl reaction volume containing 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 4 $\mu\text{g}/\mu\text{l}$ *E. coli* tRNA, labeled pNB2 RNA and 11.5 U/ μl RNase T1 (BRL). Digestion was allowed to proceed for 10 min at 37°C . RNase A digestion was done in 5 μl containing 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 4 $\mu\text{g}/\mu\text{l}$ *E. coli* tRNA, labeled pNB2 RNA and 50 $\mu\text{g/ml}$ RNase A (Sigma). Incubation was at 37°C for 1 min. RNase CL3 digestion was performed in a reaction volume of 5 μl containing 20 mM sodium- PO_4 buffer (pH 6.5), 1 $\mu\text{g}/\mu\text{l}$ *E. coli* tRNA, labeled pNB2 RNA and 0.02 U/ μl RNase CL3 (Boehringer Mannheim). The samples were incubated at 37°C for 4 min.

Before addition of enzyme all samples were incubated for 2 min in a boiling water bath, and small aliquots were removed which represented the 0 min time points. At the end of the digestion period, urca was

added and the reactions quenched on ice. The samples were heated to 95°C prior to gel electrophoresis through 19% (Figure 3A and C) or 24% (Figure 3B) polyacrylamide-7 M urea gels. The oligo(dT)₄₋₂₂ ladder was obtained from BRL.

Creation of deletion/substitution mutants

The DNA templates containing deletions or substitutions were obtained by means of recombinant PCR (Higuchi, 1990). The same two outside primers (S566 and S567) were used to obtain all four anchor deletion/substitution mutants. The forward outside primer overlapped with the *EcoRI* site in the pBluescript SK(-) vector (Stratagene) and the reverse outside primer overlapped with the *BamHI* site. This allowed cloning of the mutated PCR products directionally into *EcoRI/BamHI* double-digested vector DNA. To generate the deletion mutant $\Delta(I)$ (see Figure 1A), the primer pairs S566/S569 and S568/S567 were used in two separate primary PCR using pNB2 DNA as a template. The products of the two reactions were gel-isolated and combined to serve as a template for the secondary PCR using the outside primers S566 and S567. The PCR fragment was digested with *EcoRI* and *BamHI*, gel-isolated and cloned. The substitution mutant S(I) was generated with the inside primers S570 and S571. The double deletion mutant $\Delta(I+II)$ was obtained with the inside primers S595 and S596 and by using $\Delta(I)$ DNA as template for the primary PCR. The double substitution mutant S(I+II) was obtained with the inside primers S597 and S598, and by using S(I) DNA as a template for the primary PCR reactions. To create the deletion mutant $\Delta(5'PER)$ (see Figure 1A) only one round of PCR was required, by using the primer set S605/S567 and pNB2 DNA as template. The sequences of all recombinant clones were confirmed by DNA sequence analysis using the dideoxy nucleotide sequencing method.

Pretreatment of mitochondrial extract with micrococcal nuclease

Mitochondrial extract was incubated in a 35 μ l reaction containing 5 mM HEPES (pH 7.5), 1 mM CaCl₂ and micrococcal nuclease (MN). After 2 h at room temperature, 1 μ l 0.25 M EGTA was added. For the substrate masking control reactions, 1 μ l 0.25 M EGTA was mixed with the MN aliquot prior to addition of mitochondrial extract. After predigestion/incubation, synthetic substrate RNA and the ingredients necessary to obtain optimal U-incorporation, as described above, were added to the reaction and the total volume thereby increased to 50 μ l. The reaction was then processed as described above.

Addition of 3' deoxyadenosine (cordycepin) or UTP to the 3' ends of RNA with poly(A) polymerase

To obtain 3' addition of cordycepin, 2 μ g gel-isolated RNA were incubated at 37°C in a 25 μ l reaction containing 0.4 mM cordycepin-triphosphate (Sigma), 50 mM Tris-HCl (pH 7.9), 0.25 M NaCl, 10 mM MgCl₂, 2.5 mM MnCl₂, 0.35 mg/ml BSA and 1 U poly(A) polymerase (BRL). After 30 min, an additional aliquot of enzyme was added and the reaction allowed to proceed for another hour.

End-labeling with uridylyate was achieved by incubating 2 μ g of RNA under the same conditions as described above, but by omitting cordycepin-triphosphate and adding 50 μ Ci [α -³²P]UTP (800 Ci/mmol) and 125 μ M cold UTP. Under these conditions ~5 mol of UTP were incorporated per mol of substrate RNA.

Synthetic oligodeoxynucleotides

Oligomers were synthesized by standard methods using an Applied Biosystems 381A Synthesizer and were purified by TLC on silica gel. The following oligomers were used in these studies. The function of the oligomer is indicated after the sequence, which is shown 5' to 3': FP = forward or 5' primer, RP = reverse or 3' primer, RH = RNase H. For localization of the oligomers on the pNB2 sequence see Figure 1A. LEIKPMAX = *Leishmania tarentolae* kinetoplast maxicircle DNA sequence in GenBank. Non-encoded nucleotides needed for cloning, *in vitro* transcription or creation of an oligo(U) tail, are in parentheses.

S165: CCTAACTAAAACCTACACC (pNB2 RH, nt 5475-5456 in LEIKPMAX)
S177: ACAAATAAAGCAACTAAAAA (pNB2 RH, nt 5545-5526 in LEIKPMAX)
S178: CATGCTAAGCAAACACCACA (pNB2 RH, nt 5520-5501 in LEIKPMAX)
S244: (TAATACGACTCACTATAGGG)TTATGGTAATTAGTTACATG (ND7 FP, nt 3240-3260 in LEIKPMAX)
S245: CCTCGATGTAATACCCAATAATTAC (ND7 RP, nt 3451-3426 in LEIKPMAX)

S246: AATACGCCATGGGCTGCTGGATG (ND7 RH, nt 3379-3357 in LEIKPMAX)
S270: (TAATACGACTCACTATAAGG)ATTTTTACAAGTGTAGAAA (gND7-II FP, nt 395-376 in LEIKPMAX)
S288: (AAAAAAAAAAAA)TAATCATACTCCATAT (gND7-II RP, nt 350-364 in LEIKPMAX)
S492: TCGTGTAGTCGGCTAA (ND7 RH, nt 3312-3297 in LEIKPMAX)
S566: ATCGAATTCCTTTGCAGCCCATAC (Mutants FP)
S567: (AGTGGATCCCC)ACAAATAAAG (Mutants RP, nt 5545-5536 in LEIKPMAX)
S568: GAGAGAAAAGAAAAGCAGCAGTATATGGTGTAG [$\Delta(I)$ FP, nt 5408-5424/5446-5462 in LEIKPMAX]
S569: CTACACCATATACTCGTGCCTTTTCTTTCTCTC [$\Delta(I)$ RP, nt 5462-5446/5424-5408 in LEIKPMAX]
S570: GAGAGAAAAGAAAAGGC(AAATTGAAAGTTCAGTA)TTAT-TACGATATATGG [S(I) FP, nt 5408-5424/5441-5457 in LEIKPMAX]
S571: CCATATACTCGTAATAA(TACTGAACTTCAATTT)GCCTTT-TCTTTTCTCTC [S(I) RP, nt 5457-5441/5424-5408 in LEIKPMAX]
S595: ATAAAAGCGGAGAGAAAAGCAGCAGTATATGGTG ($\Delta(I+II)$ FP, nt 5399-5415/5423-5424/5446-5459 in LEIKPMAX)
S596: CACCATATACTCGTCTTTCTCTCCGCTTTTAT [$\Delta(I+II)$ RP, nt 5459-5446/5424-5423/5415-5399 in LEIKPMAX]
S597: ATAAAAGCGGAGAGAAA(TCTTTTC)GC(AAATTGAAGTT-CAG) [S(I+II) FP, nt 5399-5415/5423-5424 in LEIKPMAX]
S598: (CTGAACTTCAATTT)GC(GAAAAGA)TTTCTCTCCGCTTT-TAT [S(I+II) RP, nt 5424-5423/5415-5399 in LEIKPMAX]
S605: (ATCGAATTCCTGCAGCCC)AAGCGGAGAGAAAAGAA [$\Delta(5'PER)$ FP, nt 5403-5419 in LEIKPMAX]
S656: (TAATACGACTCACTATAGGG)ATATGACTTGAAGTTAAA-AG (gCYb-I FP, nt 16800-16781 in LEIKPMAX)
S657: (AAAAAAAAAAAA)AAAATTTATATATCTTTTAAAC (gCYb-I RP, nt 16767-16788 in LEIKPMAX)
S658: (TAATACGACTCACTATAGGG)CTTTTCTAAAATAATAAAAA-AAG (gCYb-II FP, nt 2291-2270 in LEIKPMAX)
S659: (AAAAAAAAAAAA)AAAATATATTTTCTCATGTTA (gCYb-II RP, nt 2237-2258 in LEIKPMAX)
S1025: AACAACTGAAGTAAA (pNB2 RH, nt 5441-5425 in LEIKPMAX)
S1111: TTATTTATTTTCTTATGC (pNB2 RH, nt 5374-5357 in LEIKPMAX)

Acknowledgements

We thank all members of the Simpson laboratory for advice and discussion. G.C.F. was supported by the Swiss National Science Foundation and by an American Cancer Society fellowship (PF-3824). This research was supported in part by research grant AI09102 from the National Institutes of Health to L.S.

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Received on August 19, 1994; revised on October 7, 1994.