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Cotranscriptional editing of *Physarum* mitochondrial RNA requires local features of the native template

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ABSTRACT

RNAs in the mitochondrion of *Physarum polycephalum* are edited by the precise cotranscriptional addition of nonencoded nucleotides. Here we describe experiments to address the basis of editing specificity using a series of chimeric templates generated by either rearranging the DNA present in editing-competent mitochondrial transcription elongation complexes (mtTECs) or linking it to exogenous DNA. Notably, run-on transcripts synthesized from rearranged mtTECs are edited at the natural sites, even when different genes are ligated together, yet exogenous, deproteinized DNA does not support editing. Furthermore, the accuracy of nucleotide insertion in chimeric RNAs argues that any *cis*-acting determinants of cytidine insertion are limited to small regions surrounding editing sites. Taken together, these observations strongly suggest that template-associated factors affect read-out of the mitochondrial genome.

Keywords: insertional RNA editing; mitochondrion; *Physarum*; transcription elongation

INTRODUCTION

Many organisms, including humans, express RNAs with specific sequence differences relative to the genes from which they are transcribed. These alterations, which include base substitutions and insertion or deletion of nucleotides, are needed to generate functional RNAs, and, in some cases, are used to produce alternative gene products and/or regulate expression of proteins (Gott & Emeson, 2000). Editing mechanisms are diverse, and can occur at the transcriptional or posttranscriptional level. Cotranscriptional editing events are particularly interesting, as they may have parallels to processes regulating transcriptional elongation.

A majority of the RNAs transcribed from the mitochondrial genome of *Physarum polycephalum* are extensively edited by the precise insertion of mono- (C, U) and dinucleotides (CU, GU, UA, AA, GC, UU) in a process that is closely linked to transcription (Gott, 2001). Insertions occur, on average, once every 25 nt in mRNAs, and once every 40 nt in rRNAs and tRNAs (Miller et al., 1993). These alterations are predicted to create open reading frames in mRNAs and conserved motifs in structural RNAs. Four specific C-to-U changes have also been observed in *Physarum* mitochondrial RNAs (Gott et al., 1993), but these appear to arise via a distinct mechanism (Gott, 2001).

Two systems have been developed to study *Physarum* insertional RNA editing in vitro: isolated mitochondria and partially purified mitochondrial transcription elongation complexes (mtTEC). Both involve analysis of nascent RNAs that are initiated in vivo and extended in vitro. Run-on transcripts synthesized in isolated mitochondria are generally fully edited (Visomirski-Robic & Gott, 1995), whereas there is only partial editing at each site in RNAs synthesized by mtTECs (Cheng & Gott, 2000). The extent of editing in both systems can be systematically varied, however, by altering the reaction conditions (Cheng et al., 2001). Studies using these systems led to the conclusion that insertional editing in *Physarum* mitochondria is a cotranscriptional process, whereby nonencoded nucleotides are added to the 3′ ends of nascent transcripts (Cheng et al., 2001).

The determinants that specify sites of insertion and the identity of the nucleotide to be added are still unknown. Because transformation methods have not yet been developed for *Physarum* mitochondria, it is not possible to systematically change sequences surrounding editing sites in vivo. Template alterations are also

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not feasible in currently available in vitro systems, as they employ run-on RNA synthesis from the endogenous mitochondrial genome. An obvious solution would be to use exogenous DNA as transcription/editing templates; however, RNAs initiated in vitro on tailed or bubble templates containing cloned mitochondrial sequences are not edited when synthesized in fractionated mitochondrial extracts (A. Majewski, E. M. Byrne, & J.M. Gott, unpubl. data). The lack of editing with artificial templates could be due to a variety of reasons, including a need for initiation from an authentic promoter, the absence of template or RNA sequences needed to signal editing or assembly, a requirement for specific DNA modifications in the template, or loss, sequestration or inefficient assembly of necessary *trans*-acting factors.

To delimit the features required for editing, we have used a series of chimeric templates that consist of editing-competent mtTECs linked to other DNA fragments, allowing run-on transcription of the added DNA by the transcription-editing machinery. Here we show that mtTECs that have been treated with restriction enzymes and ligated to other DNA fragments are still transcriptionally active and support editing. Intriguingly, we have found that although editing occurs during transcription from the endogenous mitochondrial DNA (mtDNA) in chimeric templates, RNAs synthesized from downstream, deproteinized DNAs are not edited. Remarkably, however, templates consisting of self-ligated mtTEC fragments *do* support editing both upstream and downstream of a ligation junction. These findings are most easily explained by a requirement for proteins and/or other factors associated with mtDNA for insertion of nonencoded nucleotides into nascent *Physarum* mitochondrial transcripts.

This work represents the first examination of editing patterns for individual molecules synthesized in vitro by mtTECs. We observe an interspersion of unedited and edited sites in a wide variety of patterns, providing evidence that individual sites are edited independently. Importantly, sequences derived from self-ligated mtTECs, which include large-scale rearrangements both within and between genes, restrict the location of any *cis*-acting elements to ~15–20 bp of DNA on either side of a C insertion site and roughly 15 nt of upstream RNA sequence. These data indicate that sites of cytidine insertion are specified locally and have important implications regarding the nature of the editing apparatus.

RESULTS

Generation of chimeric templates

As pre-formed mtTECs contain all factors necessary for run-on transcription and editing, we reasoned that they could be used as the source of RNA polymerase and editing factors for a series of chimeric templates in which the endogenous DNA was ligated to DNA fragments from various sources. Our goal was to separate any signals required for assembly of the transcription/editing complex from those used to direct editing, thus allowing us to define the sequences involved in specifying editing sites. The strategy used to generate chimeric templates involves digestion of mtTECs with a restriction enzyme followed by ligation to an exogenous DNA cassette (Fig. 1A). Because the DNA in mtTECs is associated with RNA polymerase and other proteins (Cheng & Gott, 2000), we first ascertained whether it was accessible to restriction enzymes. The data in Figure 1B indicate that DNA present in mtTECs is efficiently digested by *Xba*I under our conditions (lane 2), yielding a restriction pattern similar to that of deproteinized mitochondrial DNA (lane 1). Hybridization of a southern blot of this gel with an end-labeled oligonucleotide complementary to the *atp* gene resulted in a single strong band of ~900 bp in both cases (Fig. 1C, lanes 1 and 2), indicating that the mtTEC DNA was digested at both *Xba*I sites within this gene. Thus, the DNA in mtTECs can be readily digested by restriction endonucleases.

To determine whether digested DNA in mtTECs is a good substrate for ligation, aliquots were incubated with DNA ligase and different amounts of a tagged DNA cassette derived from the cloned *atp* gene. Comparison to the unligated sample revealed additional bands having mobilities consistent with cassette ligation to one (+1), or at higher cassette concentrations, both (+2) ends of the *Xba*I fragments (Fig. 1C, lanes 3–5). This cassette carries an *Xba*I-compatible overhang on one end, and a 41-bp "tag" on the other end that provides unique primer binding sites for RT-PCR analysis of the RNA products. Probing the same blot with an end-labeled oligonucleotide complementary to the tag sequence (Fig. 1D) confirmed that the tagged cassette was indeed ligated to all *Xba*I fragments. The absence of signal in lanes 1 and 2 of Figure 1D also indicated that the tag-specific probe does not hybridize to mitochondrial DNA, an important consideration in the interpretation of subsequent RT-PCR experiments. Based on these results, we conclude that the tagged cassette can be ligated to mtTEC DNA fragments quite efficiently, making it feasible to generate chimeric templates for editing experiments.

RNAs are synthesized from chimeric templates

We next examined whether this mixture of templates could be used to generate chimeric RNAs. Since there are 12 *Xba*I sites within the circular mitochondrial genome of *Physarum*, there are 24 different fragment ends that could potentially be ligated to the tagged cassette. Furthermore, because multiple genes are transcribed in mtTECs (Cheng & Gott, 2000), a number of *Xba*I

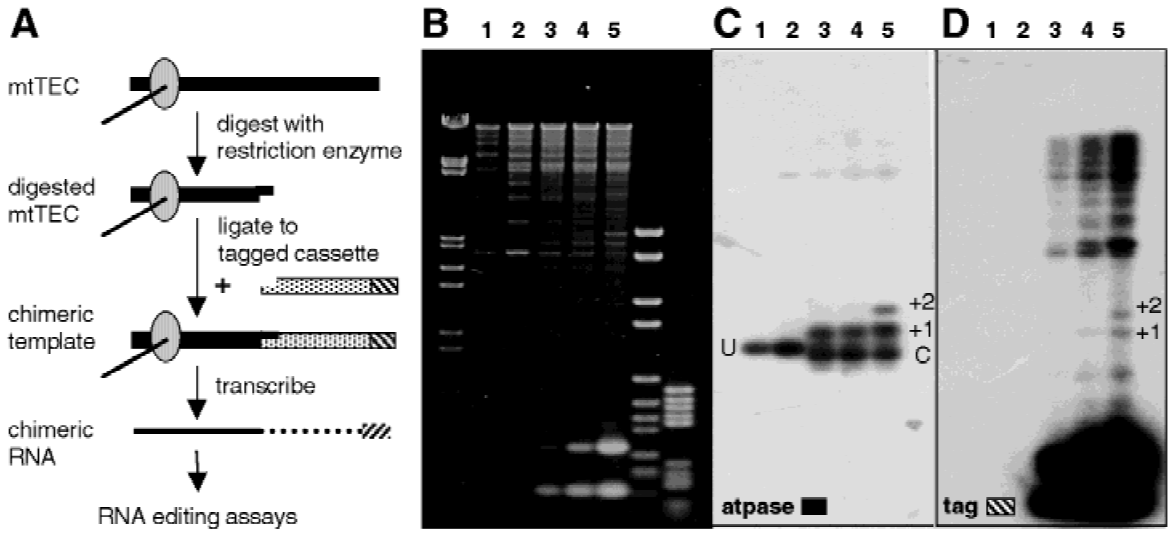

FIGURE 1. Accessibility of DNA in mtTECs. **A:** Schematic representation of the steps used to generate chimeric templates and RNAs. **B:** Electrophoresis of mitochondrial DNA fragments. Lane 1: deproteinized *Physarum* mtDNA digested with *Xba*I; lane 2: *Xba*I-digested mtTECs (11.6 μg protein); lanes 3–5: ligation of tagged cassette to digested mtTEC DNA: lane 3: 0.5 pmol; lane 4: 1.5 pmol; lane 5: 5 pmol (~2-, 6-, and 20-fold molar excess over mtTEC ends). **C:** Southern blot of gel shown in **B** probed with an *atp*-specific oligonucleotide. U: unligated fragment; +2: fragment with cassettes ligated to each end; +1: tagged cassette ligated to one end; C: circularized fragment. **D:** Southern blot of the same filter stripped and probed with a tag-specific oligonucleotide.

fragments should be associated with RNA polymerases transcribing one or both strands. We therefore used S1 nuclease protection to determine whether we could detect a transcript derived from a specific chimeric template within the complex mixture of RNAs produced upon run-on transcription.

In the experiment shown in Figure 2, a single-stranded DNA (ssDNA) containing both *atp* and tag sequences was used to protect RNAs from digestion with S1 nuclease. This probe includes not only sequences present in the tagged cassette (*atp*, stippled and tag, hatched regions), but also upstream *atp* sequences that could only be derived from the mitochondrial genome (black). Based on the sequence of this S1 probe, we would expect a protected RNA from the endogenous *atp* gene to be 261–271 nt in length (depending on the extent of editing at the 10 sites in this region), whereas the chimeric RNA fragment derived from the reconstructed *atp* gene should be longer (302–312 nt) due to the added tag sequences. Each of these RNA products was observed under appropriate conditions. In the absence of *Xba*I digestion (Fig. 2, lanes 1 and 2), an RNA product of the size expected for the endogenous *atp* mRNA was observed; this band was lost upon *Xba*I digestion (Fig. 2, lanes 3–5). Conversely, protection of an RNA of the size predicted for the "reconstruction chimera" was dependent upon *Xba*I digestion, inclusion of the tagged DNA cassette, and DNA ligase (Fig. 2, lane 5), as expected. Thus, active mtTECs are substrates for both cleavage and ligation and maintain elongation competence throughout this entire procedure.

We also expected to see a shorter protected RNA species derived from transcription of tagged cassettes ligated to other *Xba*I fragments present in digested mtTECs. Because the upstream regions of such RNAs

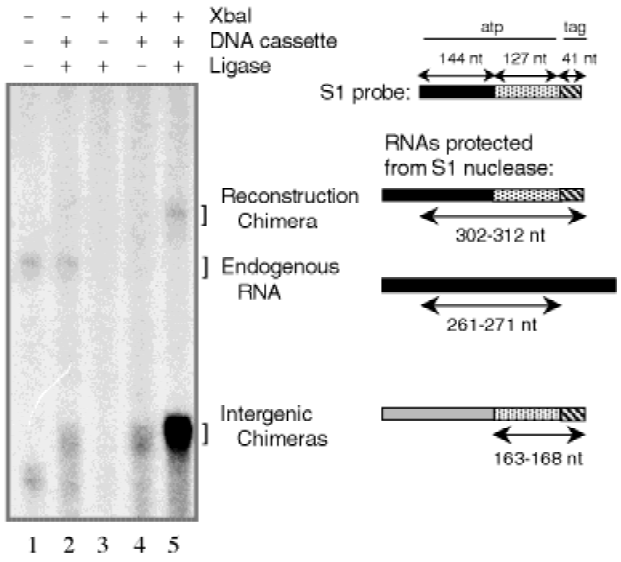

FIGURE 2. RNAs are synthesized from chimeric templates. MtTECs (19.5 μg protein ± 3.8 pmol cassette DNA) were treated as described prior to run-on transcription in the presence of 5 μM GTP/25 μCi α-^{32}P-GTP, then chased with 500 μM GTP for 10 min. RNAs were protected from S1 nuclease using a ssDNA containing both *atp* and tag sequences; regions derived from the endogenous *atp* gene are shown in black; those from other regions of the genome in gray. Portions of chimeric RNAs derived from the tagged cassette are stippled (*atp*) or hatched (tag). Arrows indicate the regions of the endogenous and chimeric RNAs protected by the ssDNA probe.

(light gray) would not be protected by the S1 probe used in these experiments, each of the "intergenic chimera" RNAs should yield a protected RNA fragment of 163–168 nt depending on whether editing occurs downstream of the ligation junction. An RNA of this size could also arise by initiation of transcription from the end of the added DNA cassette and, indeed, we did observe some signal of this size in all reactions containing the tagged cassette (Fig. 2, lanes 2, 4, and 5). However, because this band was much more abundant under conditions that allow for the production of chimeric DNAs (Fig. 2, lane 5), the vast majority of the 163–168 nt RNA in lane 5 is likely to have been derived from intergenic chimeras.

Intergenic chimera transcripts are not edited in regions downstream of the ligation junction

We next asked whether the labeled RNAs produced by run-on transcription of intergenic chimeras (IGC) were edited. S1-protected IGC RNAs were eluted, digested with RNase T1, and electrophoresed on a 20% gel as in Visomirski-Robic and Gott (1997). As expected, these protected IGC transcripts (Fig. 3, lane 1) lacked sequences upstream of the junction (italics), but did contain RNase T1 fragments encoded in the tagged cassette. Nearest neighbor analyses of these T1 fragments gave results consistent with their proposed identities (data not shown). The absence of bands c′ and d′ (arrowheads), which are diagnostic of editing at two sites downstream of the junction, clearly indicates that IGC RNAs are not edited at these sites. In contrast, bands comigrating with the edited fragments were present in control experiments in which a tagged cassette containing an edited version of the *atp* gene was used (data not shown), providing evidence that the exogenously supplied DNA is accurately transcribed in each case. We also employed a version of the RT-PCR assay described below to analyze IGC transcripts, with results entirely consistent with the foregoing conclusions (data not shown).

Chimeric RNAs are edited only in regions synthesized from endogenous mtDNA

The lack of editing in regions of the IGC RNAs derived from the tagged DNA cassette indicated that, although the starting mtTEC preparations were editing-competent, the editing machinery did not seem to be operative after passing into the exogenous DNA in these chimeras. There are a number of possible reasons for this difference, including inactivation of the editing machinery or the absence of native sequences upstream that are required for editing at downstream sites. To address these possibilities, we examined the extent of editing both upstream and downstream of the junction

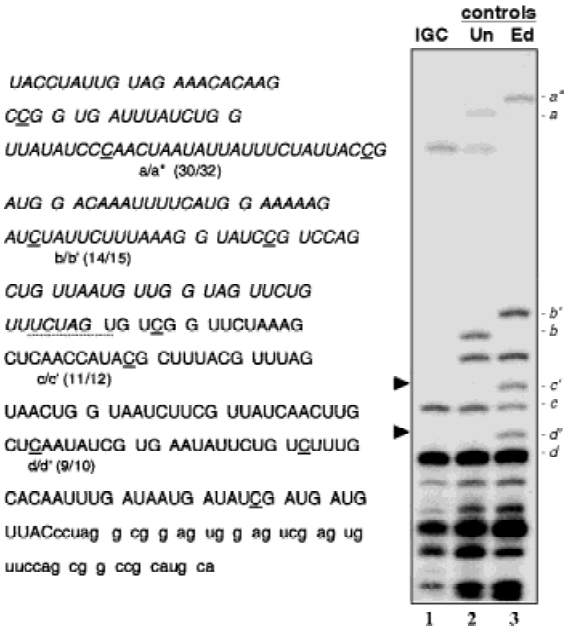

FIGURE 3. Regions of the intergenic chimera RNAs corresponding to the tagged cassette are not edited. Labeled RNAs were generated by run-on transcription of chimeric templates (126 μg TEC protein/38 pmol cassette) at 5 μM GTP/600 μCi α-^{32}P-GTP followed by a 10-min chase with 500 μM GTP and protected as described in Figure 2. S1-protected RNAs derived from intergenic chimeras (IGC; lane 1) and control transcripts containing either genomic (Un: unedited, lane 2) or cDNA (Ed: edited, lane 3) sequence were isolated, digested with RNase T1, and electrophoresed. The sequence of the protected region is shown at the left; nucleotides in italics are not included in the tagged cassette and are therefore not present in intergenic chimeras. Tag sequences are shown in lower case letters, the junction region is denoted by a dotted underline, and editing sites are indicated by underlined Cs. Large oligonucleotides that contain sites of C insertion are labeled with lower case letters (a/a″), with each prime (′) sign indicating an added nucleotide. Fragment sizes in unedited and edited control transcripts are given in parentheses. Arrowheads indicate the absence of RNase T1 fragments diagnostic of editing within IGC RNAs.

in transcripts from the reconstruction chimera. This fusion results in the exact reconstitution of the *atp* gene except for a single nucleotide change (A to T) at the junction and the addition of the tag at the 3′ end of the template. These changes provide a means of distinguishing between the endogenous and reconstructed genes and allow selective RT-PCR amplification of the chimera-derived RNAs.

To measure the extent of editing within the pool of run-on transcripts from reconstruction chimeras, RNA was reverse transcribed using the outer tag-specific primer, then subjected to PCR using an upstream *atp*-specific primer and an internal 5′ end-labeled tag-specific primer. The resulting end-labeled PCR fragment was then digested with restriction enzymes that distinguish between edited and unedited sequences (Fig. 4A). In this experiment, the RT-PCR products, which are dependent on both run-on transcription and reverse transcription (data not shown), migrate as a doublet. The lower band most likely represents completely un-

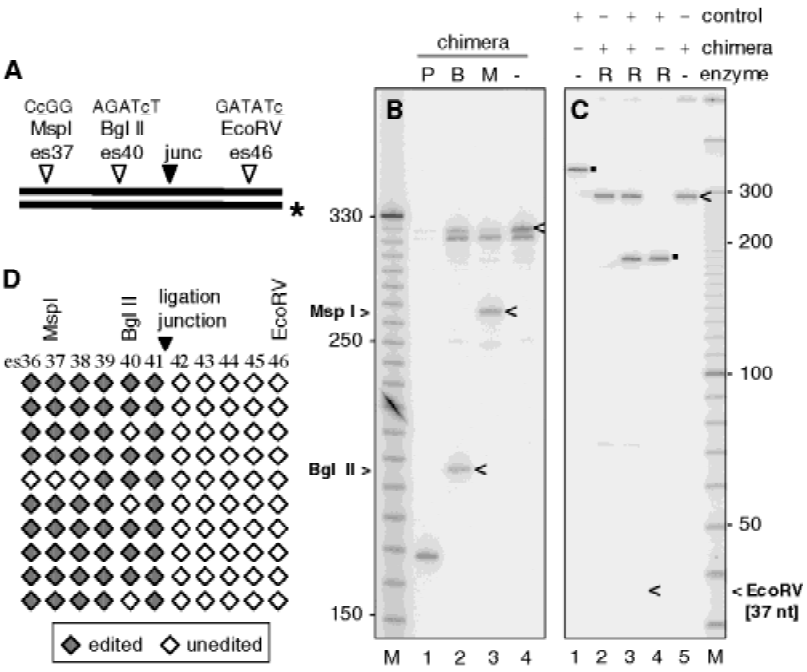

FIGURE 4. Reconstruction chimera RNAs are edited upstream, but not downstream, of the junction. **A:** Schematic representation of the RT-PCR fragment analyzed in **B** and **C**, with the junction (junc) and restriction sites used to assess the extent of editing at editing sites (es) 37, 40, and 46 indicated. Sites of C insertion within each recognition sequence are indicated by an underlined, lower case c. PCR primers were 13atp and end-labeled PCRH1. Restriction digests of RT-PCR fragments derived from reconstruction chimera RNAs are shown in **B** and **C**. Chimeric RNAs were synthesized using 9 μg mtTEC/1.5 pmol cassette as template; transcriptions included 5 μM GTP, followed by a 10-min chase with 500 μM GTP. Arrowheads indicate the positions of the uncut PCR product and the fragments expected upon editing at sites monitored by *Msp*I, *Bgl*II, and *Eco*RV. **B:** 4% gel: lane 1, P: *Pvu*II digestion (site present in cDNAs from both edited and unedited RNAs); lane 2, B: *Bgl*II; lane 3, M: *Msp*I; −: undigested PCR products. **C:** 8% gel: lane 1: control PCR fragment (generated from mtDNA with 24cytb and 5′-labeled 5cytb) prior to digestion; lane 2: chimera RT-PCR product + *Eco*RV (R); lane 3: mixture containing both control and chimera products + *Eco*RV; lane 4: control fragment + *Eco*RV, lane 5: untreated chimera product. Dots indicate the positions of uncut and cut control fragments. **D:** Schematic representation of the extent of editing at 11 C insertion sites (es 36–es46) within individual sequenced cDNA clones generated by RT-PCR of reconstruction chimera RNAs. A shaded diamond represents the presence of an inserted C residue at that editing site; a diamond with no shading indicates that the clone was unedited at that site. Sequences that were unedited at all positions are not shown. Restriction sites assayed in **B** and **C** are indicated for reference.

edited RNA; unedited sequences have been found among cloned products from this and other run-on experiments (data not shown) and by direct analysis of labeled run-on RNAs (A. Majewski & J.M. Gott, unpubl. data). The upper band represents partially edited RNA, with 76% of the cDNAs cut by *Msp*I, indicative of C insertion at editing site 37 (es37), and 51% digested with *Bgl*II (diagnostic for editing at es40) (Fig. 4B, lanes 2 and 3). In contrast, no editing was observed at es46, which lies downstream of the ligation junction, as evidenced by the lack of a 37-nt fragment in lanes 2 and 3 of Figure 4C (see arrowhead). To rule out trivial explanations for the lack of digestion, mixing experiments were carried out to confirm that *Eco*RV was active under these conditions; the control DNA was digested with *Eco*RV (Fig. 4C, lane 3). Therefore, we conclude that the failure to cleave the pool of chimeric cDNA molecules was due to lack of editing at es46.

To determine the extent and pattern of editing at each insertion site within individual transcripts, RT-PCR products derived from reconstruction chimera RNAs were also cloned and sequenced. Each of these clones contained tag sequences and the point mutation adjacent to the junction, indicating that they were derived from reconstruction chimeras rather than endogenous mRNAs. The editing status at each C insertion site examined is shown in Figure 4D. Editing sites upstream of the junction were extensively edited, with some molecules displaying unedited patches, consistent with incomplete editing of RNAs synthesized in vitro as previously observed (Cheng & Gott, 2000). Because unedited sites are presumably transcribed in vitro, the fact that they can be followed by edited sites before the junction strongly suggests that editing can take place during run-on transcription after cleavage and ligation. Importantly, however, we see no evidence of editing at sites downstream of the junction, even though the reconstruction chimeras contain natural upstream sequences.

Finally, to further characterize the pattern of editing, we also carried out direct RNase T1 analysis of the labeled S1 nuclease-protected transcript from the reconstructed gene (data not shown). Again we found that editing sites upstream of the junction were partially edited, providing additional evidence that editing can occur after the cleavage and ligation procedures (confirmed below). However, we saw no evidence of editing downstream of the junction in the reconstruction chimera, consistent with results from both cDNA sequencing and RT-PCR experiments. We therefore conclude that editing does not occur on DNA templates derived from cloned *Physarum* genes, despite the fact that these templates have the same nucleotide sequence as mitochondrial DNA. A caveat is the single base change at

the 5′ end of the added ~160-bp cassette. However, it seems unlikely that this change could be responsible for disrupting editing at the five downstream editing sites even if upstream sequences are important for editing, especially in light of experiments described below.

Production of rearranged DNA templates from mtTEC preparations

The rather surprising result that RNA transcribed from exogenous DNA is not edited, even in its correct sequence context, led us to explore other means of generating altered templates that support editing in vitro. Possible reasons for the lack of editing in regions downstream of the junction include (1) inactivation of the editing machinery upon passage through the ligation junction, (2) a requirement for some distinguishing characteristic of mtDNA, such as the presence of specific covalent modifications, and/or (3) the necessity for proteins or other factors bound to the mtDNA. To distinguish among these possibilities, we examined transcription from one region of mtDNA into another in the absence of added DNA.

The data shown in Figure 1C suggested that mtDNA fragments circularize efficiently in the presence of DNA ligase, yielding an *atp* band (C in lanes 3–5) on a native gel that migrates faster than the linear species and does not hybridize to the cassette probe (Fig. 1D). To verify the production of these rearranged DNAs, we digested mtTECs with *Xba*I and ligated the resulting fragments in the absence of exogenous DNA (Fig. 5A). The existence of DNA molecules containing ligated junctions was demonstrated by PCR, using primers that do

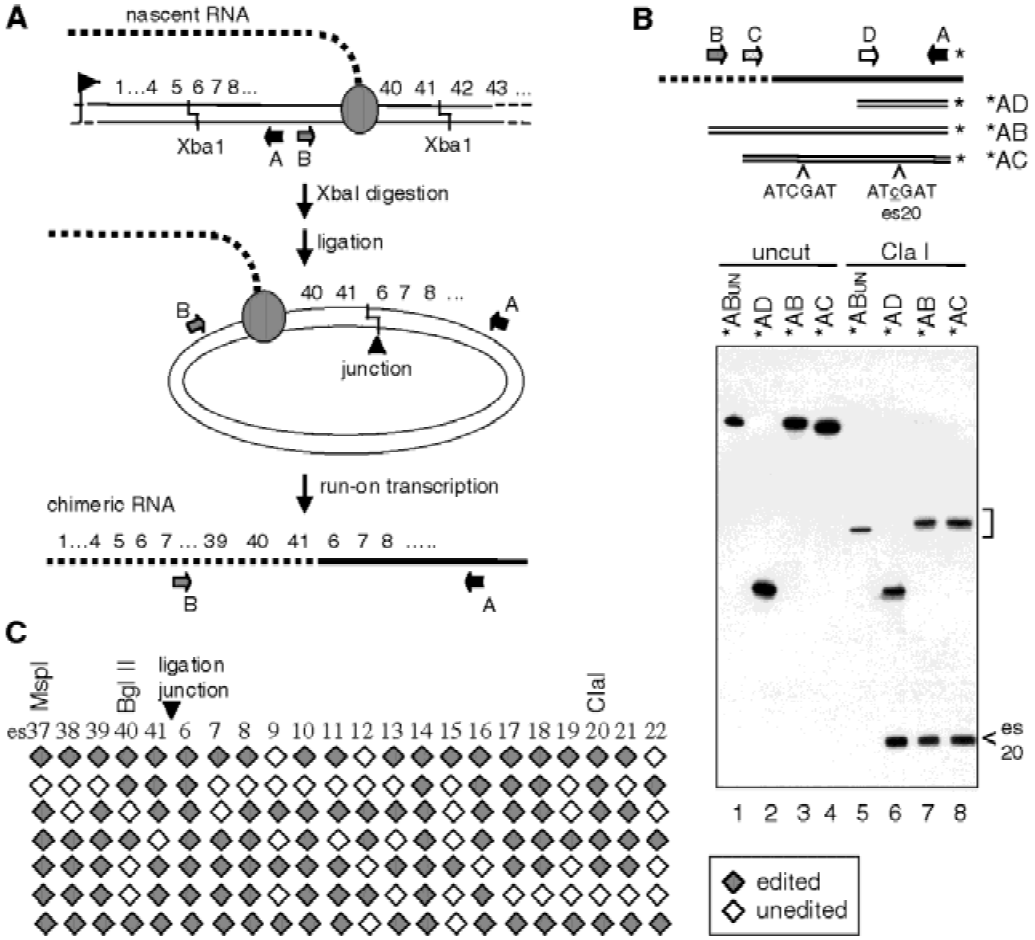

FIGURE 5. RNAs from circularized templates are edited both upstream and downstream of the junction. **A:** Schematic representation of the steps used to generate circular *atp* templates and chimeric RNAs. Arrows A and B represent PCR primers used to confirm chimera formation. **B:** Analysis of RT-PCR products on a 4% gel. The *Cla*I restriction site (AT$\underline{C}$GAT) used to assess the extent of editing at *atp* es20 is indicated; RT-PCR products AB and AC contain a second, upstream *Cla*I site that is present in cDNAs derived from both edited and unedited RNAs (bracket). Uncut (lanes 1–4) and *Cla*I-digested (lanes 5–8) PCR products generated with end-labeled primer A and primer B, C, or D as noted. Lanes 1 and 5: PCR product from the circular DNA template (unedited), lanes 2–4 and 6–8: RT-PCR products generated from run-on transcripts synthesized at 500 μM GTP. **C:** Schematic representation of editing at 22 C insertion sites within individual cDNA clones generated by RT-PCR of chimeric RNAs using primers 19atp and 27atp. Symbols as in Figure 4.

not yield a PCR product on native mitochondrial DNA due their "outward" orientations relative to one another (arrows A and B in Fig. 5A). Only after digestion and self-ligation were PCR products of the expected size observed (data not shown).

Transcripts derived from circularized mtTEC fragments are edited downstream of the junction

The efficiency of circularization allowed us to create transcription templates in which regions of mtDNA were rearranged, with editing sites normally upstream now present downstream of the junction. After determining that these chimeric molecules were transcribed, using S1 nuclease protection (data not shown), we subjected the chimeric RNAs to RT-PCR analysis. As observed previously, the editing sites upstream of the junction were extensively edited, as assayed by *Msp*I and *Bgl*II digestion of bulk RT-PCR products (data not shown). However, transcripts derived from circular mtDNA templates were also edited to a significant extent at sites *downstream* of the ligation junction (Fig. 5B). Insertion of a C at *atp* es20 results in the generation of a second *Cla*I recognition site (Fig. 5B, lanes 6–8) that is not present upon PCR amplification of the circular DNA template (Fig. 5B, $*AB_{un}$, lane 5). Thus, the appearance of an additional *Cla*I band in samples derived from chimeric RNAs indicates that nucleotide insertion must have occurred during run-on synthesis, because the extra C is not present in the DNA template.

To confirm that chimeric RNAs were edited at es20 and to determine whether nucleotides were also added at other editing sites downstream of the junction, individual RT-PCR clones were sequenced. Consistent with our analysis of bulk RT-PCR products, substantial editing was observed both upstream *and* downstream of the junction (Fig. 5C). Interestingly, individual molecules had interspersed unedited and edited sites in a range of patterns. Similar results were observed with an intergenic circular template in which *atp* sequences were linked to sequences from the gene encoding the small subunit of rRNA (*ssu*), which is also edited (Fig. 6 and Byrne & Gott, in prep.). These data demonstrate that transcription across a ligated junction does not abolish editing, ruling out the idea that inhibition of editing in RNA transcribed from non-mitochondrial DNA is due to the presence of the restriction enzyme, DNA ligase, or a residual nick. It should also be noted that, although we refer to the templates for these chimeric RNAs as being circular based on the efficiency of circularization observed in Figure 1, these transcripts could, in principle, also be derived from chimeras formed from two separate molecules having the same sequence. Regardless of the precise nature of the template, the results indicate that ligation of one region of mtTEC DNA to another creates a chimeric template that supports RNA editing throughout its entire length.

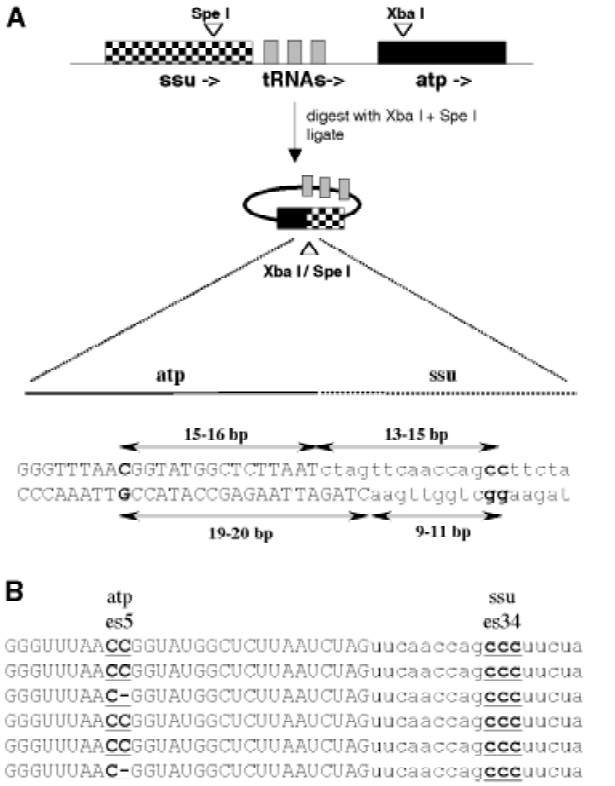

FIGURE 6. Editing occurs close to the ligation junction. **A:** Schematic representation of the *ssu/tRNA/atp* region of the *Physarum* mitochondrial genome with pertinent restriction sites noted. Arrows indicate the direction of transcription for each gene. Circular templates were generated as in Figure 5A, except that mtTECs were digested with *Xba*I + *Spe*I prior to ligation. Predicted *atp* (*Xba*I)/*ssu* (*Spe*I) junction, with upstream *atp* (upper case) and downstream *ssu* (lower case) sequences shown. The distance between es5 (*atp*) and es34 (*ssu*) and the junction are shown independently for the template and nontemplate strands (ranges are given because Cs are inserted next to encoded Cs (bold) at each site). **B:** Sequences of cDNA clones spanning the *atp-ssu* junction in chimeric RNAs synthesized at 500 μM GTP; RT-PCR using primers 7atp and 10ssu. Ambiguity regarding the site of C insertion is indicated by underlining.

Editing determinants are located close to C insertion sites

The DNA molecules in the mtTECs analyzed in Figure 5 contain a rearrangement within the same gene, such that the central section of *atp* is revisited by the transcription/editing machinery in the absence of both the 5′ and 3′ ends of the gene. Importantly, even the sites adjacent to the junction are edited, suggesting that ~30–35 bp of native DNA downstream and 12–18 bp upstream of an editing site are sufficient to support C insertion. To further test the DNA requirements for editing specificity, we also juxtaposed sequences from two different genes by digesting mtTECs with both *Xba*I and *Spe*I before self-ligation. Circular-

ization then joins the 5′ end of the *atp* gene to the 3′ portion of the *ssu* gene, excluding the upstream region of the *ssu* gene and the downstream segment of the *atp* gene (Fig. 6A). Sequences surrounding the junction region in cDNA clones derived from run-on transcripts of this circle are shown in Figure 6B. Significantly, four of six sequences show editing at the upstream *atp* site and all are edited at the downstream *ssu* site. In this chimera, *ssu* es34 is preceded by only 9–11 nt of the *ssu* gene on the template strand and 13–15 nt on the nontemplate strand, whereas *atp* es5 is followed by only 19–20 nt of natural *atp* sequence on the template strand and 15–16 nt on the nontemplate strand. Thus, these results unequivocally demonstrate that any *cis*-acting template determinants for C insertion are localized to ~15 bp of upstream and ~20 bp of downstream DNA.

The results in Figure 5 also suggest that C insertion does not require *cis*-acting RNA signals more than 18 nt upstream of *atp* es6. However, because the entire upstream *atp* RNA sequence is present immediately 5′ of the repeated section in these chimeric RNAs, this experiment does not rule out a role for long-range interactions involving upstream RNA. The data from the intergenic rearrangement of Figure 6 demonstrate that no more than 15 nt of local RNA sequence are needed for editing at *ssu* es34, and suggest that distal upstream RNA sequences are unlikely to contribute editing determinants. Because the start sites of most mitochondrial transcripts have not yet been identified in *Physarum*, it is formally possible that the *ssu* and *atp* genes are cotranscribed. However, long transcripts containing *ssu* and *atp* sequences were not observed on northern blots by Miller and colleagues (Jones et al., 1990) and, even if present in our chimeric transcripts, any upstream *ssu* RNA sequences would be displaced by at least an additional ~1,300 nt consisting of the original 3′ *ssu* sequence, three tRNAs, and the 5′ end of *atp*. Therefore, the involvement of interactions between distal upstream RNA and the transcription/editing machinery appears unlikely. Taken together, our data argue that any *cis*-acting nucleic acid determinants required for insertion of nonencoded C residues are local.

Deproteinized mitochondrial DNA does not support editing upon ligation to mtTECs

To test whether some distinguishing characteristic of mtDNA, such as the presence of specific covalent modifications, was responsible for the observed differences in the ability of templates to support editing, we next asked whether DNA isolated from mitochondria could support editing when ligated to cleaved mtTECs (Fig. 7A). In these experiments, we digested deproteinized mitochondrial DNA and isolated a specific *Avr*II-*Eco*NI restriction fragment derived from the cytochrome b (*cytb*) gene for ligation to *Xba*I-digested mtTECs. This fragment was of particular interest because it encompasses a CG insertion site (*cytb* editing sites 3 and 4), a U insertion site (es11), and a site of CU insertion (es12/es13), in addition to nine C insertion sites. Analysis of RT-PCR products from the *atp*/*cytb* chimera revealed that editing occurred at sites up-

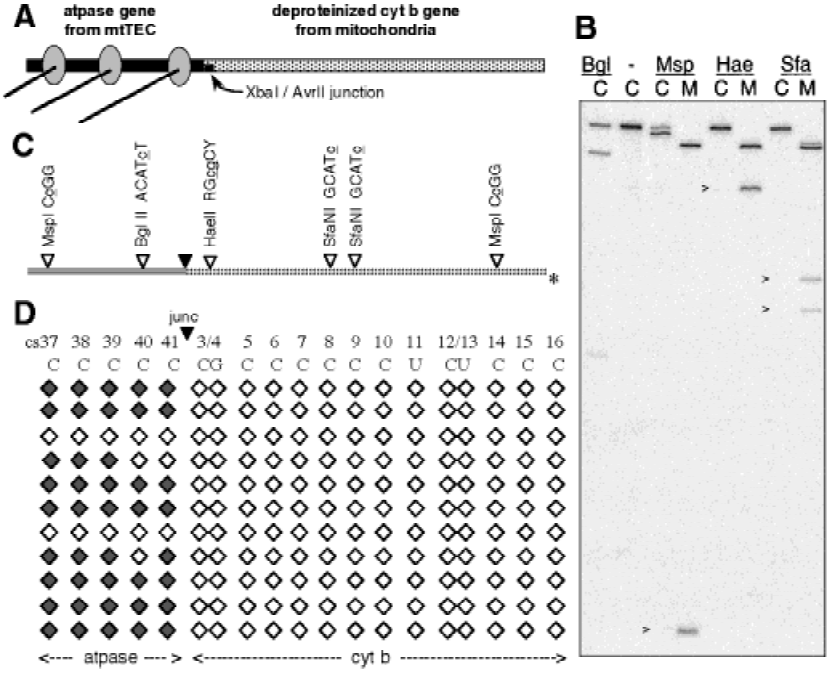

FIGURE 7. Deproteinized mitochondrial DNA does not support editing. **A:** Schematic representation of the chimeric template. **B:** Restriction digests of gel-purified RT-PCR products derived from mitochondrial control (M) and chimeric (C) RNAs. Chimeric RNAs were generated by transcription at 500 μM GTP (5 μg TEC protein/0.6 pmol *cytb* DNA); RT-PCR using primers 24cytb and 19atp. The control was generated by RT-PCR of mitochondrial nucleic acids using 24cytb and 5cytb. Arrowheads indicate the size of end-labeled fragments expected upon editing at sites monitored by *Msp*I, *Hae*II, and *Sfa*NI digestion. **C:** Schematic representation of the RT-PCR fragment analyzed in **B**, with the *atp*/*cytb* ligation junction and restriction sites used to assess the extent of editing at *atp* sites 37 and 40, and *cytb* sites 3/4, 8, 9, and 14 indicated. **D:** Schematic representation of editing status at the CG, U, CU, and C insertion sites within sequenced cDNA clones generated by RT-PCR of chimeric RNAs. Symbols as in Figure 4.

stream, but not downstream of the junction (Fig. 7B, D and data not shown). For example, although *Msp*I sites are created in the cDNA upon editing at both *atp* es37 and *cytb* es14, cleavage by *Msp*I was only observed at the upstream site. The same pattern was observed upon sequence analysis of individual cDNA clones (Fig. 7D). Thus, in the absence of associated proteins and/or other factors, DNA isolated from mitochondria does not support editing under conditions that yield edited RNAs from self-ligated mtTECs.

DISCUSSION

Editing patterns within individual molecules

We have previously shown that bulk RNA synthesized by mtTECs is partially edited (Cheng & Gott, 2000) and that editing efficiency at individual sites is influenced by relative nucleotide concentrations and local sequence context (Cheng et al., 2001). This work extends those observations by examining editing patterns of individual RNA molecules. The cDNA sequences demonstrate directly that edited and unedited sites are interspersed, and that C insertion at any one site does not depend upon any simple pattern of editing upstream (Figs. 4–7 and data not shown). In addition, as described in a separate paper (Byrne et al., in prep.), a low level of misediting occurs during run-on RNA synthesis in both native and rearranged mtTECs, but insertion of the incorrect nucleotide does not preclude accurate editing at downstream insertion sites. In aggregate, these data confirm inferences from previous work that (1) individual sites are edited independently of one another, and (2) that the partial editing observed in mtTECs is not due simply to the loss of an editing factor associated with a subset of individual transcription complexes during isolation of mtTECs. It should be noted, however, that although most mtTECs in our preparations are editing-competent, the loss of associated editing factors could account for the subset of completely unedited transcripts observed in these and other experiments.

The *cis*-acting signals that direct cytidine insertion are local

The studies reported here were motivated, in part, by a desire to change sequences around editing sites to define the *cis*-acting elements that specify editing. Although we have not made systematic nucleotide changes in these experiments, our large-scale rearrangements do allow us to draw a number of conclusions regarding regions that might be involved in selection of C insertion sites. First, based on our observations that nonencoded cytidines are efficiently added at editing sites immediately upstream of ligated junctions, any specific downstream DNA editing determinants must occur within ~15–20 bp of an insertion site. For example, normal levels of C insertion occur at *atp* editing site 41 even when downstream DNA is contributed from (1) cloned *atp* sequence that does not itself support editing (Fig. 4), (2) deproteinized *cytb* DNA isolated from mitochondria (Fig. 7), or (3) mtTEC DNA derived from regions of the *atp* gene that normally lie upstream of this region (Fig. 5). Similarly, *atp* es5 is also readily edited in chimeras containing downstream DNA from cloned *coI* or noncontiguous *atp* sequences (data not shown), or regions of the *ssu* gene derived from mtTEC DNA (Fig. 6). These results are consistent with the finding that when mtTECs are digested with a restriction enzyme that removes DNA 14 bp downstream of an editing site prior to transcription, the run-off RNAs produced are still edited at that site (A. Majewski & J.M. Gott, unpubl. data).

The second major conclusion from these studies is that editing-site selection requires only a limited region of specific DNA upstream of C insertion sites. Results from chimeras involving rearranged mtTEC DNA fragments demonstrate that, remarkably, the template does not have to be transcribed in the natural order for editing to occur at a given site. This is illustrated by the fact that editing occurs at *atp* es6 in the intragenic TEC rearrangement of Figure 5 and at *ssu* es34, which is joined to a different position within the *atp* gene in the intergenic rearrangement of Figure 6. The latter experiment demonstrates that any specific upstream DNA determinants required for C insertion must be located within approximately 15 bp of the insertion site.

Third, the results presented here suggest that specific nascent RNA sequences farther than ~15 nt upstream of the site of editing are unlikely to be required for C insertion. This conclusion is based upon both the experiment shown in Figure 5, where only 18 nt of native RNA sequence are present immediately upstream of *atp* es6, and the *atp*-*ssu* chimera data shown in Figure 6. In the latter case, editing occurs at *ssu* es34 despite the fact that the sequence immediately upstream of the junction in the nascent transcript is contributed from the *atp* gene, with only 13–15 nt of upstream RNA having the naturally occurring *ssu* sequence. These data argue against a role for specific distal RNA elements in identification of C insertion sites. Thus, it is unlikely that elements analogous to the long-range interactions observed in RNA- (Sen et al., 2001) or protein- (VanGilst et al., 1997) mediated antitermination are involved in editing. We also infer from these data that there are unlikely to be *trans*-acting editing factors for C insertion that associate with/dissociate from the transcription-editing machinery in a gene- or sequence-specific fashion.

The constraints these data place on the location of any *cis*-acting elements required for editing are also consistent with the fact that interspersion of unedited and misedited sites in RNA does not preclude editing at

downstream sites. Indeed, the localized nucleic acid requirements for editing are not surprising given that nontemplated nucleotides are added to mRNAs every 25 nt on average, and frequently occur within 12–14 nucleotides of each other (Miller et al., 1993).

Template sequences alone are insufficient to specify nucleotide insertion

The experiments presented here indicate that insertional RNA editing does not occur on exogenous DNA. This was true whether assaying labeled run-on RNA directly by S1-protection (Fig. 3), or indirectly by restriction enzyme digestion of bulk RT-PCR products (Figs. 4C and 7B) or sequence analysis of individual clones (Figs. 4D and 7D). In cases where we have ligated PCR fragments generated from cloned *Physarum* genes to mtTEC DNA followed by run-on transcription, the regions of RNA transcribed from the exogenously added DNA are entirely unedited, both in the context of intergenic chimeras (Fig. 3) and the reconstruction chimera (Fig. 4). Up to the tag, the reconstruction chimera differs from the natural *atp* sequence by only a single base pair adjacent to the ligation junction. Thus, the cloned DNA has been placed in an essentially native sequence context, yet its editing sites are not utilized. These results argue strongly that additional factors are necessary.

Apart from conventional base sequence, are there other characteristics of the mtDNA that are involved locally in specifying the positions and identity of nucleotide insertion, or more globally in initiating or maintaining the editing activity? There are, for example, precedents for DNA modifications altering transcription dynamics. Eukaryotic cytosine methylation affects transcription initiation (Attwood et al., 2002) and elongation (Rountree & Selker, 1997), and 5-hydroxymethyl cytosines in bacteriophage T4 DNA prevent the phage *alc* termination protein from acting upon the *Escherichia coli* RNA polymerase (Kashlev et al., 1993). A preliminary mass spectroscopic study of *Physarum* mtDNA indicated that, although no unusual bases were detected, low levels of 5-methyl cytosine are present (J.M. Gott & P. Crain, unpubl. data). We therefore tested whether naked mtDNA would support editing by creating chimeric templates in which an isolated fragment of deproteinized mtDNA was ligated to digested mtTEC DNA (Fig. 7). None of the 14 insertion sites within the ligated mtDNA fragment were edited in chimeric RNAs, despite the fact that editing was observed within the same transcript in regions upstream of the junction. Thus, although we cannot rule out a requirement for DNA modifications in editing, the inability of deproteinized mtDNA to support insertion of nonencoded cytidines, uridines, or dinucleotides indicates that DNA containing naturally occurring covalent modifications cannot direct editing on its own.

DNA-associated factors are most likely required for RNA editing in *Physarum* mitochondria

Given that rearranged mtTECs support editing, but deproteinized mtDNA in a similar context does not, it seems likely that editing requires DNA-associated proteins or factors. There are many systems in which nucleic acid templates function in the context of a nucleoprotein complex that is traversed by polymerases. The chromatin structure of eukaryotic nuclear genomes modulates both transcription initiation and elongation by RNA polymerases, largely regulated by the interplay between histone modifying enzymes and ATP-dependent chromatin remodeling complexes (Workman & Kingston, 1998). A second, editing-related example is provided by the paramyxoviruses, which edit their P gene mRNA cotranscriptionally. In this case, the viral polymerase slips at a single, precisely determined site, resulting in the addition of one or more pseudotemplated G residues to the nascent RNA (Hausmann et al., 1999). Transcription of the paramyxoviral genome, which occurs in the form of a helical ribonucleoprotein particle, requires the nucleocapsid protein (Kolakofsky et al., 1998). Thus, despite the fact that the *Physarum* mitochondrial RNA polymerase can transcribe naked DNAs both in the context of elongation across chimeric templates (Figs. 3, 4, and 7) and after initiation on tailed or bubble templates generated via PCR (A. Majewski, E. M. Byrne, & J.M. Gott, unpubl. data), it is possible that such a nucleoprotein template is required for editing site recognition and/or utilization.

Nucleic acid templates present in nucleoprotein complexes exhibit a range of accessibilities to chemical and enzymatic probing. The bases in the genomes of segmented negative strand RNA viruses, for instance, are reactive to modifying agents, and the backbone is susceptible to cleavage by RNase T1 and S1 nuclease, despite the presence of bound N protein, which is required for transcription (Klumpp et al., 1997). Thus, our finding that the DNA present in mtTECs is accessible to both restriction enzymes and DNA ligases is compatible with the presence of bound proteins or other factors.

Finally, *Physarum* mitochondrial DNA is found in a nucleoid structure composed of mitochondrial DNA, proteins, and RNA (Suzuki et al., 1982). Given that the circular mitochondrial genome is normally in a highly condensed state, editing might require a particular template topology. The data presented here render this possibility unlikely. DNA from *Physarum* mitochondria becomes nicked during isolation (A. Rhee & J.M. Gott, unpubl. data) and thus the DNA in mtTEC preparations is probably relaxed. Although individual topological domains may remain intact in these mtTEC preparations, the fact that templates derived by self-ligation of linearized mtTECs support the same extent of editing as untreated mtTECs (Figs. 5 and 6; data not shown)

argues that a highly supercoiled template is not required. In addition, editing can occur on linear templates: labeled run-off transcripts from mtTECs that have been cut close to editing sites with a restriction enzyme (but not ligated) contain inserted nucleotides (A. Majewski & J.M. Gott, unpubl. data) and linear chimeric templates support editing upstream of the junction (Figs. 4 and 7 and data not shown). Although precedents exist for the generation of superhelical torsion even on linear templates (e.g., the SWI/SNF complex; Havas et al., 2000), taken together, our data suggest that topological strain is unlikely to play a crucial role in nucleotide insertion.

Implications for RNA editing in *Physarum* mitochondria

Here we have demonstrated that *Physarum* mitochondrial editing requires some feature of the native transcription template that can be removed by procedures that deproteinize DNA, and that only a small region of the native template and upstream RNA sequence is needed to specify an editing event during transcription. These results suggest that, in addition to the polymerase and any editing components that may travel with it, nucleotide insertion requires template-associated proteins or nucleic acid constituents which do not move with the polymerase. How might they function? These factors might simply coat the DNA nonspecifically or interact selectively with editing site determinants. Such factors could act to enable an editing activity associated with the polymerase to function, for example, by slowing transcription to allow the competing process of nontemplated insertion to occur at editing sites (Cheng et al., 2001). Alternatively, or in addition, these factors might be involved directly in demarcating editing sites, perhaps even specifying the nucleotide to be inserted. Indeed, unedited and misedited sites in run-on transcripts may be due to dissociation of such factors in vitro. Our most immediate goal is to identify the specific features of the template and/or factors required for editing.

MATERIALS AND METHODS

Oligodeoxynucleotides

T7: 5′-AATACGACTCACTATAG-3′. *atp* gene: 3atp, AACAA AACATGCTGCTGCT; 7atp, TCAACGTTATCTTTTGAATT CAG; 8atp, CAATGTTAACTGGTTATAAAA′; 9atp, AGGCAC TTTGTGCAGCG; Avr12atp, ATCCTAGGAGTAATAAAATTA AAAGC; 13atp, CTAATTTCGGTGGAGGTTC; Spe11atp, GGGACTAGTGTGGTTCTAAAGCTCAAC; Avr18atp, TAAG AATTCCTAGGGTAACATCATC; 19atp, GGGGTACCTATTG TAGAAACACA; 27atp, CCAATTCCAACATACACAC. *cytb* gene: 5cytb, CAGTGATATGCTTATTTAAGGACAT; 24cytb, GTAGCTGCCCAATAAGACAT. *ssu* gene: 10ssu, TCCATGG TAATAATGGTCCAGCAGCAG. Tag-specific: RVTH1, TGCAT GCGGCCGCTGGAA; PCRH1, CACTCGACTCCACTCCGC; HCT1, TGCATGCGGCCGCTGGAACACTCGACTCCACTC CGC; HCN1, CTAGGCGGAGTGGAGTCGAGTGTTCCAGC GGCCGCATGCA.

Plasmids

pHCatp11-12U: PCR was performed on genomic sequence with primers Spe11atp and Avr12atp. The product was digested with *Avr*II, ligated to tag DNA (annealed HCT1/HTN1), digested with *Sph*I, and cloned into pBSM13+ between *Sma*I and *Sph*I. pHCatp11-12E: as for pHCatp11-12U, but with cDNA sequence as PCR template. pHCatp11-18U: the PCR product generated from pHCatp11-12U with T7 and Avr18atp was digested with *Spe*I and *Avr*II, and used to replace the longer *Spe*I-*Avr*II insert of pHCatp11-12U. pHCatp11-18E: as for pHCatp11-18U, but using pHCatp11-12E as template. pHCatp19-18E: *atp* cDNA fragment from 19atp to the second *Xba*I site cloned into pHCatp11-18E between *Sma*I and *Spe*I, generating a *Xba*I-*Spe*I hybrid site. pHCatp3-18E: as for pHCatp19-18E, except that *atp* sequence extends to 3atp. pHCatp3-18U: as for pHCatp3-18E, except that the upstream *atp* fragment is unedited.

Preparation of DNA cassettes

The *atp* cassette was generated by PCR from plasmid pHCatp11-18U using the RVTH1 and T7 primers, digestion with *Spe*I (NEB) and purification from an agarose gel. mtDNA was isolated using the Qiagen Genomic Tip 100 Midi system and digested with *Eco*NI and *Avr*II (NEB). The *cytb* fragment used in Figure 7 was purified from an agarose gel, extracted with phenol and chloroform/isoamyl alcohol, and ethanol precipitated.

MtTEC manipulations

mtTECs were isolated essentially as described in Cheng and Gott (2000) with minor variations in dialysis conditions. Typically, mtTEC DNA was digested by incubating mtTECs (2–9 μg protein) with 15–30 U μL *Xba*1(NEB) in the presence of 1$\times$ Buffer H (Roche) in a final volume of 30–45 μL at 30 °C for 15–20 min. The reactions in Figures 7B and 6 also contained 15 U *Hin*cII (NEB) and *Spe*I (NEB), respectively. Ligations were carried out at 16 °C for 30 min with 500 μM ATP, 1.4 U T4 DNA ligase (Roche), and exogenous cloned DNA where appropriate. Run-on transcription reactions (45–50 μL) were similar to those described previously (Cheng & Gott, 2000) except for higher levels of Tris (43–50 mM, pH 7.8) and NaCl (67–90 mM). GTP concentrations varied and are noted in the figure legends. For Figures 4–7, two rounds of DNase I digestion/BioSpin P30 column (Biorad) were performed prior to RT-PCR.

Southern hybridization analysis

After *Xba*I digestion and subsequent ligation, mtTECs were treated with RNase A and deproteinized. DNA fragments were separated on a 1.5% agarose gel, stained with ethidium bro-

mide, then transferred to Genescreen (DuPont NEN). The blot was probed with 5′-end-labeled 13atp, stripped, and re-probed with labeled HCN1.

S1 nuclease protection and RNase T1 digestion

Run-on RNAs were subjected to three rounds of S1 nuclease digestion (Cheng & Gott, 2000) using 4 μg (first round) or 3 μg protecting ssDNA derived from pHCatp19-18E. Gel-purified S1-protected RNAs were digested with RNaseT1 and fragments separated on a 20% acrylamide denaturing gel. Control RNAs for Figure 3 were produced using the Ambion Maxiscript In Vitro Transcription kit and *Not*I-linearized pHCatp3-18U and pHCatp3-18E, followed by a single round of S1 nuclease protection with 1 μg ssDNA.

Reverse transcription

RNAs were annealed to 2.5 pmol primer in 10 μL by incubation at 70 °C for 10 min, then on ice for 10 min, and reverse transcribed at 48 °C for 60 min in a final volume of 20 μL using 100 U M-MLV Reverse Transcriptase (RNase H minus; Promega), supplied buffer, and 0.5 mM dNTPs, followed by RNase digestion. Primers were RVTH1 (Fig. 4), 9atp (Fig. 5), 27atp (Fig. 5), 10ssu (Fig. 6), and 24cytb (Fig. 7).

PCR

PCR was performed over 30 cycles (1 min at 94 °C, 1 min at 50 °C, and 2 min at 72 °C) with a 7-min final extension, using 2.5 U Taq DNA polymerase (Roche) per 100 μL in buffer supplied by the manufacturer, with 200 μM dNTPs. End-labeled PCR products for restriction enzyme analysis were generated in 50-μL reactions, using 25 pmol cold oligo and 12.5 pmol 5′-end-labeled oligo. For sequencing, RT-PCR products were cloned in pBSM13 (Stratagene). Reactions in Figure 5 were performed with primers A (9atp) and B (13atp), and the gel-purified product was re-amplified using end-labeled A, with primers B, C (19atp), or D (8atp).

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