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Evolution of four types of RNA editing in myxomycetes

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ABSTRACT

The myxomycete *Physarum polycephalum* requires extensive RNA editing to create functional mitochondrial transcripts. The cytochrome c oxidase subunit 1 (*coI*) transcript exhibits a combination of editing forms not found together in any other eukaryotic RNA: 66 insertions of ribonucleotides (59 Cs, a single U, and three mixed dinucleotides) as well as base conversion of four Cs to Us (Gott et al., *J Biol Chem*, 1993, *268*:25483–25486). Through a phylogenetic survey of *coI* DNA genes and RNA transcripts in representative myxomycetes, we have decoupled the four types of editing in this lineage. Some myxomycetes share insertional editing with *P. polycephalum*, yet lack C → U conversion, consistent with previous reports of separation of insertional and base conversion editing in *P. polycephalum* extracts (Visomirski-Robic & Gott, *RNA*, 1995, *3*:821–837). Most remarkably, we detect unique evolutionary histories of the three different types of insertional editing, though these have been indistinguishable in vitro. For example, *Clastoderma debaryanum* exhibits insertions of Us, but not Cs or dinucleotides.

Keywords: acellular slime mold; base conversion; cytochrome oxidase; mitochondria; phylogeny; *Physarum polycephalum*

INTRODUCTION

"RNA editing" is the alteration of RNA sequences by base modifications, substitutions, insertions, or deletions. Since the initial discovery of extensive U insertions and deletions in trypanosome mitochondrial mRNAs (Benne et al., 1986), many additional and apparently unrelated examples of editing have been described in organisms ranging from Ebola virus to humans. Myxomycetes (acellular slime molds) are the only group of organisms that combine multiple types of editing within the same transcript. For example, *Physarum polycephalum*'s 1.5-kb transcript encoding cytochrome c oxidase subunit 1 *(coI)* is modified by 59 C insertions, a single U insertion, three dinucleotide insertions, and even four sites of C → U base conversion (Gott et al., 1993).

Though *coI* is the only *P. polycephalum* RNA known to undergo base conversion editing, the insertional editing found in this transcript is typical of almost all mitochondrial RNAs in *P. polycephalum*. Mitochondrial mRNAs, tRNAs, and rRNAs of *P. polycephalum* all require similar patterns of insertional editing to create functional transcripts. Most editing events are cytidine insertions, but there are also a small number of specific and reproducible insertions of uridines and dinucleotides. The insertional editing sites are sprinkled throughout the transcripts, never closer than 9 nt. The average spacing of insertions is 40 nt apart in ribosomal RNAs and 25 nt apart in messenger RNAs. Though no consensus sequence for edited sites has been detected, C insertions have a preference for following purine/pyrimidine dinucleotides, and are often in the third codon position (Miller et al., 1993).

C-to-U conversion editing of *coI* does not occur in pulse labeling experiments using isolated mitochondria under conditions that exhibit insertional editing, implying that there are separate mechanisms or components for base conversion and insertional editing (Visomirski-Robic & Gott, 1995). Biochemical evidence is less clear regarding a possible difference between single and dinucleotide insertions. Wang et al. (1999) used an amplification and restriction cutting technique to show that unedited sites of dinucleotide insertion were found within the same molecules that had complete, accurate C and U single nucleotide insertional editing. They also found two misedited clones with CC and UU at a CU insertion site, but detected no clones containing sites of single nucleotides inserted into a dinucleotide insertional site. These results were inter-

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preted to mean that dinucleotide insertional editing occurs by a process unlike single nucleotide insertions. In contrast, Visomirski-Robic and Gott (1997a) detected molecules with single Cs inserted at a CU insertion site when UTP levels in mitochondrial extracts were low, indicating that dinucleotides might be added by two paired insertions of single nucleotides.

Evolutionary analysis of insertional and base conversion editing in the mitochondria of two other lineages reveals two very disparate patterns of editing distribution. Insertional editing in kinetoplastids has diminished over time, with the earliest-diverging kinetoplastids displaying more extensive editing than the later-diverging species (Landweber & Gilbert, 1994; Maslov et al., 1994). However, analysis of base conversion editing in plant mitochondria revealed that levels of RNA editing correlated with G/C content rather than phylogenetic position (Malek et al., 1996). The pattern suggests that RNA editing in plants suppresses mutations that disrupt coding sequences. The myxomycete system provides a rich opportunity to explore patterns of both insertional and substitutional editing in the same gene and the same lineage. Here, we study the evolution of RNA editing in the *coI* transcripts of myxomycetes by examining the patterns of RNA editing in four unexplored species. We observe independent evolutionary histories of insertional editing and C-to-U base conversion editing. Remarkably, we also detect separate evolutionary origins of the three different types of insertion editing (C insertion, U insertion, and dinucleotide insertion).

RESULTS AND DISCUSSION

Gene amplification and analysis

We used the polymerase chain reaction to amplify DNA and cDNA copies of a conserved 1.2-kb region of the *coI* gene in four myxomycetes: *Stemonitis flavogenita, Didymium nigripes, Arcyria cinerea*, and *Clastoderma debaryanum* (Fig. 1) (GenBank AF239221–AF239228). Comparison of the cDNA and DNA sequences for each species revealed sites of RNA editing, shown in Table 1. Like *P. polycephalum*, all four species edit the *coI* mRNA by U insertion. In contrast, the other two forms of insertional editing are not universally present. *S. flavogenita* and *D. nigripes* both have approximately the same degree of C and dinucleotide insertional editing as *P. polycephalum*. *A. cinerea* has only a single C insertion, and *C. debaryanum* has neither C nor dinucleotide insertional editing in this region. C-to-U conversion editing is found in only two of the four investigated species, *D. nigripes* and *A. cinerea*.

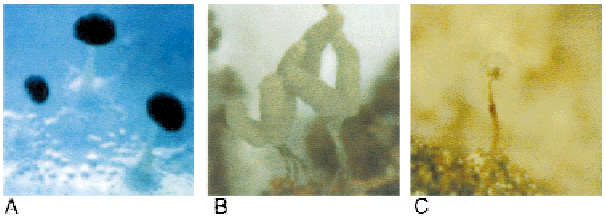

FIGURE 1. Fruiting bodies of myxomycetes: (**A**) *Didymium nigripes*, (**B**) *Arcyria cinerea*, and (**C**) *Clastoderma debaryanum*. Each structure is approximately 0.1–1 mm.

TABLE 1. Edited sites in 1.2 kb of the *coI* transcript.

	U insertions	C insertions	Dinucleotide insertions	C-to-U conversions
P. polycephalum	1	35	3	4
D. nigripes	2	40	3	4
S. flavogenita	4	34	3	0
A. cinerea	4	1	0	2
C. debaryanum	4	0	0	0

The observation that C-to-U conversion editing does not correlate with insertional editing is consistent with previous experiments that separated these editing forms in vitro (Visomirski-Robic & Gott, 1995). However, our phylogenetic survey revealed what biochemical analysis did not: the events classified together as “insertion” editing—insertion of Us, Cs, and dinucleotides—may require distinct factors or be cued by different signals, as they each appear to have emerged at separate times during evolutionary history.

Though morphology-based classification schemes for myxomycetes are controversial (e.g., Ross, 1973), a molecular phylogeny of the myxomycete group is not available. To conduct a phylogenetic analysis of editing, we amplified and sequenced portions of the nuclear small-subunit ribosomal RNA gene (*SSU*) from each organism. Using primers based on conserved regions of the gene, we obtained the complete (approximately 2 kb) *SSU* sequence from *S. flavogenita* and *D. nigripes* genomic DNA (GenBank AF239229 and AF239230).

The source of *A. cinerea* and *C. debaryanum* precluded recovery of the complete sequences of the *SSU* genes from these species. As neither of these myxomycetes is amenable to laboratory culture, they were obtained from field collections; thus the extracted nucleic acids contained exogenous material from naturally associating organisms. Amplification of some regions of the myxomycete DNA was presumably inhibited by the overwhelming presence of fungal and other DNA. However, we were able to amplify a 1.3-kb segment of the *SSU* gene from *A. cinerea* and a nonoverlapping 0.47-kb region from *C. debaryanum* (GenBank AF239231 and AF239232). The source of these sequences is unambiguously slime mold, as inferred by the high similarity to the other myxomycete sequences in both conserved and semivariable regions. Using both parsimony and

maximum likelihood methods, we constructed two phylogenetic trees based on the two regions of *SSU* rDNA sequence, as shown in Figure 2. Although we cannot determine the relative branching order of *A. cinerea* and *C. debaryanum*, we can infer that both diverged separately from the *P. polycephalum/D. nigripes/S. flavogenita* clade.

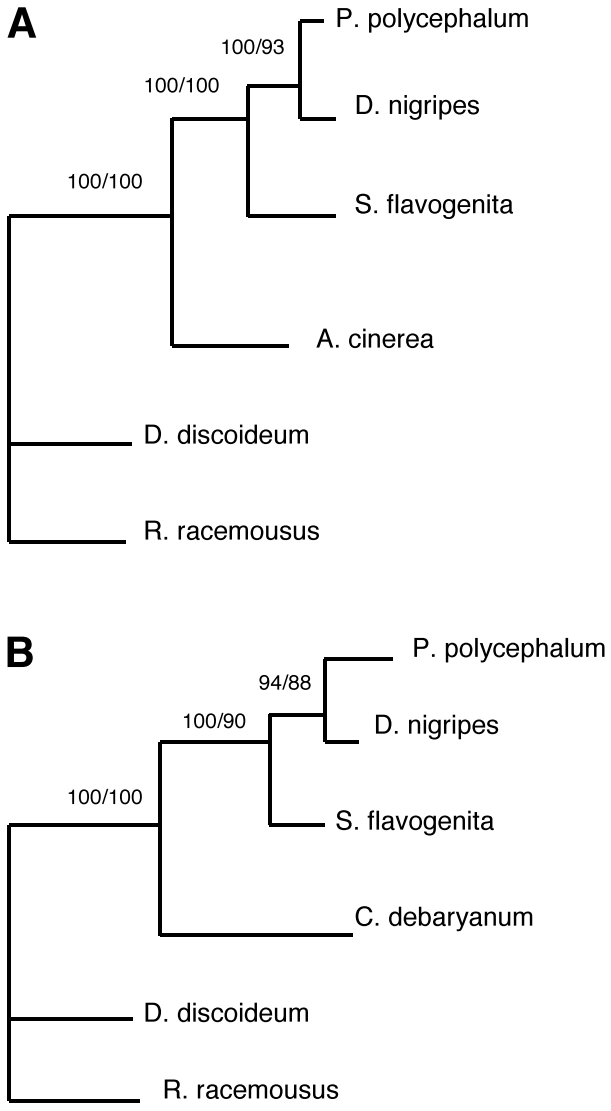

FIGURE 2. SSU rDNA phylogeny of myxomycetes. **A**: The optimal tree obtained by parsimony analysis of SSU rDNA in the region sequenced for *A. cinerea*, based on 176 informative sites. The tree is 557 steps long, and branches are drawn to scale. **B**: The optimal tree obtained by parsimony analysis of SSU rDNA in the region sequenced for *C. debaryanum*, based on 76 informative sites. The tree is 226 steps long, and branches are drawn to scale. For both **A** and **B**, bootstrap values of 100 bootstrap replicates are indicated above the nodes, with the values for parsimony analysis to the left and maximum likelihood method to the right. Outgroup taxa used to root the trees are *Dictyostelium discoideum* (X00134), a cellular slime mold, and *Rhizomucor racemousus* (X54863), a fungus. A fungal sequence was chosen as a second outgroup based on Baldauf and Doolittle (1997). Sequences for *S. flavogenita*, *D. nigripes*, *A. cinerea*, and *C. debaryanum* are deposited in GenBank (AF239229, AF239230, AF239231, AF239232). *P. polycephalum* sequence is from GenBank (X13160).

Origins of insertional editing

By examining the types of editing found in each species of myxomycete and mapping these data onto the phylogenetic framework, we can infer the relative order in which the various types of RNA editing arose in the slime molds. The presence of U insertional editing in all species examined implies that it is ancestral within the myxomycete lineage. Conservation of one particular site of U insertional editing in *C. debaryanum*, *D. nigripes*, *S. flavogenita*, and *P. polycephalum* suggests that it was edited in the last common ancestor of all these species (Fig. 3).

The absence of C insertions in the *C. debaryanum coI* sequence suggests that C insertional editing arose later than U insertions. *A. cinerea* has only a single C insertion, which is striking given that 30–40 Cs are inserted in the same region of this transcript in later diverging slime molds. We confirmed that this single C insertion was actually due to RNA editing, rather than a PCR or cloning-induced artifact, by performing a second, independent round of PCR, cloning, and sequencing on the *A. cinerea* DNA and cDNA. The results were consistent with a single site of C insertion in the RNA relative to the DNA.

Though 63–70% of C insertions in our data set are in the third codon position, the single C insertion in *A. cinerea* is not. This C insertion creates either the first or second position of the CCN codon for proline, an amino acid that is one of the least commonly substituted amino acids in protein evolution. *D. nigripes* and *P. polycephalum* also share this editing site in the homologous proline codon. Although *S. flavogenita* and *C. debaryanum* do not edit this site, their DNA sequences encode a CCN proline codon at this position, underscoring the importance of preserving this amino acid. This proline is also conserved in the cytochrome c oxidase proteins of *Dictyostelium discoideum*, plants, algae, *Acanthamoeba castellanii*, and other non-myxomycete protists. Three of the four U insertion sites in *A. cinerea coI* lie in the third codon position. The placement of the

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          644                                                 683
aci_pep   L  S  L  F   V  W  A   V  L  I   T  A  I  L
aci_rna   TTAAGTTTAT TTGTATGGGC AGTTCTTATA ACCGCTATTC
cde_pep   I  S  L  F   V  W  A   I  V  I   T  A  F  L
cde_rna   ATCTCTCTCT TTGTCTGGGC TATTGTTATA ACTGCTTTTC
dni_pep   L  S  L  F   V  W  A   V  L  I   T  V  V  L
dni_rna   TTATCTTTAT TTGTTTGGGC TGTGTTAATA ACTGTTGTAT
sfl_pep   L  S  L  F   V  W  A   V  L  V   T  V  I  L
sfl_rna   TTATCTTTAT TTGTTTGGGC AGTATTAGTA ACAGTTATAC
ppo_pep   L  S  L  F   V  W  A   V  L  V   T  V  I  L
ppo_rna   TTATCTTTAT TTGTTTGGGC TGTATTAGTA ACTGTGATTT
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FIGURE 3. Portion of *coI* alignment showing shared site of U insertional editing in four of five myxomycete species. aci: *A. cinerea*; cde: *C. debaryanum*; dni: *D. nigripes*; sfl: *S. flavogenita*; ppo: *P. polycephalum*; pep: inferred translation; rna: cDNA sequence. The *P. polycephalum* sequence is from Gott et al. (1993). Gray boxes indicate U insertions not present in the DNA sequence. The first position of the alignment (T) corresponds to nucleotide 644 in GenBank AF239221.

fourth site of U insertion is ambiguous because it is inserted next to an encoded U residue (in the sequence AA**U** UCU or AAU **U**CU, where **U** is the inserted nucleotide), but it could also be inserted in the third codon position. Therefore, *A. cinerea*'s single C insertion is its only site in these data located in a position where the predicted protein sequence unambiguously depends upon the identity of the inserted nucleotide. We return to the implications of this particular C insertion later in the discussion.

Dinucleotide insertions were found in the *P. polycephalum/D. nigripes/S. flavogenita* clade, but were absent from the *coI* transcript of the other two myxomycetes. Notably, the locations and identity of the dinucleotides inserted were exactly preserved in the three species that shared dinucleotide editing. This high degree of conservation is greater than that of other insertionally edited sites. In pairwise comparisons, these three species show a lower identity of C and U insertional editing sites than overall RNA sequence similarity (80% vs. 86% for *P. polycephalum*/*D. nigripes*, 78% vs. 85% for *D. nigripes*/*S. flavogenita*, and 59% vs. 83% for *P. polycephalum*/*S. flavogenita*). In kinetoplastids, the presence of RNAs edited only at their 5′ and/or 3′ ends has suggested a reverse transcription-mediated mechanism for loss of editing (Landweber, 1992). Without evidence of such processes occurring in myxomycetes, "loss" of any edited site, with respect to the ancestral myxomycete, might occur by random insertional mutation in the DNA genome to restore the original reading frame and obviate the requirement for RNA editing. Once a site of dinucleotide editing has been established it should be more difficult to lose than single C or U insertion sites. The chance of *two* insertional mutations occurring in a single gene within a close enough distance for tolerable frame recovery must be vanishingly small.

Nonrandom positions of C insertional editing sites

A previous study of *P. polycephalum* editing (Miller et al., 1993) identified three main characteristics of insertionally edited sites. They are spaced an average of 25 nt apart in mRNAs, but never closer than 9 nt. Many follow purine/pyrimidine dinucleotides, and they are often in the third codon position. We find that this distribution of C insertional editing sites is shared in *D. nigripes* and *S. flavogenita*. Inserted nucleotides in these two species lie an average of 23 and 26 nt apart, respectively. Most striking is the consistent lack of sites (other than dinucleotide insertions) closer than 9 nt. Any distance greater than 9 nt appears to be permissible without any discernable periodicity (maximum 82 nt observed).

C insertions in our data set occur at nonrandom positions within codons. In chi-squared analysis of insertions that can be unambiguously assigned to first, second, or third codon positions, both *D. nigripes* and *S. flavogenita coI* editing sites show statistically significant deviation ($p < 0.01$) from random. For *D. nigripes*, almost all of the deviation is due to a lower than expected frequency of insertions in second positions. For *S. flavogenita*, the deviation is almost equally due to third position insertions occurring more frequently and second position insertions occurring less frequently than expected. Presumably these findings are not indicative of characteristics of the editing mechanism, but rather reflect the evolutionary preservation of editing sites that minimize disruption of protein sequence. Hence, silent "corrections" of mRNA sequences in the third codon position would be more common than nonsynonymous insertions in the second codon position.

In a chi-squared test for association, *D. nigripes* and *S. flavogenita coI* sequences show a significant ($p < 0.001$) preference for C insertional editing sites following a purine/pyrimidine dinucleotide. Among all C-containing codons, this preference leads to a nonrandom distribution of editing sites within codons (significant in chi-squared analysis at a level of $p < 0.001$), mostly due to a preponderance of editing in ACC (threonine) and ATC (isoleucine) codons. Other sequence determinants of editing may exist near C insertion sites. *D. nigripes* has a higher than expected frequency of T nucleotides three positions before C insertion sites ($p < 0.05$ in chi-squared test for association), though the analogous frequency is not significant in *S. flavogenita* and *P. polycephalum*.

The purine/pyrimidine preference and restricted spacing of editing sites cannot be sufficient signals for editing. There are hundreds of purine/pyrimidine dinucleotides not followed by edited sites, and not all edited sites are preceded by a purine/pyrimidine dinucleotide. In a previous analysis, Mahendran et al. (1994) mapped edited sites of the *P. polycephalum* small subunit rRNA onto its predicted secondary structure, but found no correlation of editing sites with structural motifs. However, if insertional editing is truly cotranscriptional, as implied by in vitro analysis (Visomirski-Robic & Gott, 1997a, 1997b), the important structures are not those of the complete, folded RNA molecule. Rather, small, local, and transitory structural elements that form as the polymerase synthesizes the RNA may determine sites of editing.

Multiple losses and gains of C-to-U conversions

C-to-U conversions are found in the *coI* transcript of *P. polycephalum*, *D. nigripes*, and *A. cinerea*, yet are absent from *C. debaryanum* and *S. flavogenita*. This pattern can only be explained by invoking multiple losses or gains of RNA editing in this lineage. Either the ancestral myxomycete used base conversion editing in

coI transcripts, and this character was lost in *C. debaryanum* and *S. flavogenita*, or the last common ancestor lacked base conversion editing and it subsequently arose in both *A. cinerea* and the *D. nigripes*/*P. polycephalum* clade. Scenarios involving lability of C-to-U editing are quite plausible, as this form of editing has evolved independently in at least five other eukaryotic lineages. C-to-U conversion is common in plant mitochondrial and chloroplast RNAs (Pring et al., 1993), present in mammalian nuclear apolipoprotein B transcripts (Hersberger et al., 1999), and used in both marsupials and trypanosomes to increase decoding capacity of mitochondrial tRNAs (Janke & Pääbo, 1993; Alfonzo et al., 1999). In addition, the cellular slime mold *D. discoideum* edits a single C to a U in its mitochondrial small subunit ribosomal RNA (Barth et al., 1999). The enzymatic activity required for C-to-U editing may arise by duplication and divergence of biosynthetic deaminases to accommodate RNA transcripts in the active sites. APOBEC-1, the human C-to-U deaminase enzyme, is part of a larger family of cytidine deaminases and shares many structural features with enzymes that deaminate single cytidine nucleosides in biosynthetic pathways (Navaratnam et al., 1998).

The positions of C to U conversions within myxomycete sequences are quite different than placement of insertional editing sites. In contrast to the conservation of insertional sites, none of the 10 sites of C-to-U conversion is shared in any of the three myxomycetes, though all but one lie in a central 60-nt region of the sequences. Furthermore, 9 of the 10 conversions are in the first or second codon position (Fig. 4). This placement of base conversion sites in first or second codon positions is also observed in plant mitochondrial sequences, where most C-to-U editing creates nonsynonymous changes in amino acid sequence (Schuster et al., 1993). In the myxomycete *coI* alignment, each site of C-to-U editing aligns with an encoded T in the DNA sequence of the other four species examined. Seven of the 10 sites of C-to-U conversion are clustered in RNA regions corresponding to crucial helices that bind the oxygen-reducing heme-copper center in the cytochrome oxidase protein (Schafer et al., 1996). Thus the effect of sites of C-to-U conversion in myxomycetes and plants is similar: they suppress DNA mutations at critical positions in the mRNA. However, sites of C-to-U editing should be easily lost by simple transition mutations of C-to-T in the genomic copy. In plants the level of C-to-T transitions at edited sites is four times higher than the normal rate of transitions at nonedited Cs (Shields & Wolfe, 1997), implying possible selection for loss of editing in this system. Similarly, the two myxomycete species without base conversions may have lost such ancestral sites by C-to-T transition mutations.

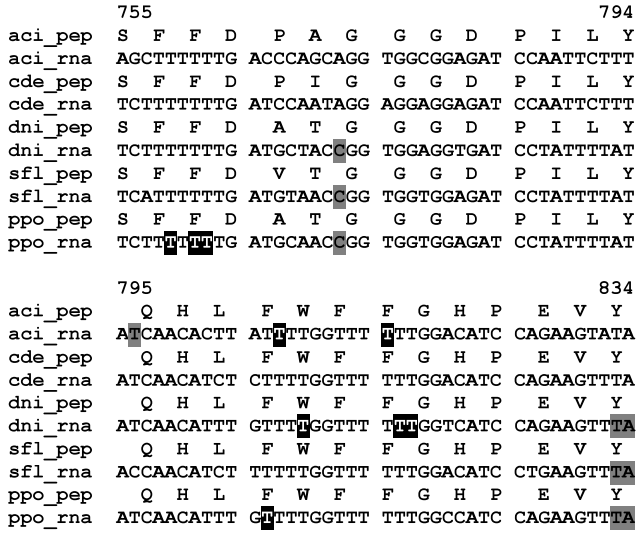

FIGURE 4. Portion of *coI* alignment showing 9 of 10 C-to-U base conversion edit sites. Sequence names are as in Figure 3. Black boxes with white letters indicate C-to-U edited sites, encoded as C in the DNA sequence. Gray boxes indicate inserted nucleotides absent from the DNA sequence. The first nucleotide of the alignment (A/T) corresponds to position 755 in GenBank AF239221.

Implications of editing distribution

This study provides the first broad survey of the distribution of RNA editing within the myxomycete lineage. Significantly, we find that the evolution of RNA editing involved changes in both the quantity and identity of inserted nucleotides. We show that myxomycete insertional editing likely arose first as occasional U additions. Only later did the capacity evolve to insert other nucleotides, eventually becoming dominated by C insertions, which currently comprise the majority of RNA editing events in *P. polycephalum*, *D. nigripes*, and *S. flavogenita*. In vitro work suggests that *P. polycephalum* editing is coupled to transcription (Visomirski-Robic & Gott, 1997a, 1997b), and thus the diversification from a mechanism that exclusively inserted Us to a repertoire that included Cs probably came with addition of specificity factors to the editing complex. A strong impetus for this adjustment in specificity might have been a case like the single C insertion in *A. cinerea*. Though the organism's primary form of RNA editing is U insertion, in one particular instance a U nucleotide would not suffice to repair a mutation in the mitochondrial genome. Factors that could rescue this mutant by promoting a C insertional event over U insertion would permit survival of the organelle with this editing specificity and lead to fixation of a mechanism for C insertion.

Why the C insertions eventually outnumbered the U insertions is not evident. The editing machinery may have a preference for CTP, or free CTP may be present at higher local concentration in the organelle. Because most of the insertions are in the third codon position, there is freedom for the editing mechanism to fluctuate

toward a preferred nucleotide. In fact, we detected two sites where such an alteration in editing specificity may have occurred. A C insertion shared by *P. polycephalum*, *D. nigripes*, and *S. flavogenita* occurs at a homologous position to a site of U insertion in *C. debaryanum* (Fig. 5), and a site of C insertion in *D. nigripes* is possibly homologous to a site of U insertion in *S. flavogenita* (site 444 of *coI* sequence alignment). These sites may be representative of the pattern that drives changes in insertional editing in myxomycetes over evolutionary time.

Because of the lack of resolution of the phylogenetic relationship between *A. cinerea* and *C. debaryanum*, an alternative interpretation of the data is possible. If these two organisms are actually sister taxa, rather than two independent basal branches of the myxomycete lineage, then an equally parsimonious scenario would be loss of the ancestral insertional editing characters in a later-diverging *A. cinerea/C. debaryanum* clade. However, without evidence for the shared common ancestry of *A. cinerea* and *C. debaryanum* to the exclusion of the other myxomycetes, this interpretation lacks support.

Although insertional editing in myxomycetes shows a progressional change in repertoire of editing mechanisms and numbers of edited sites through time, the existence of C-to-U conversion has been more ephemeral. Conversion editing of *coI* has been gained or lost at least twice in the *coI* transcript in myxomycetes, although it remains to be tested whether C-to-U editing or any other editing form is present in the genes we did not explore. However, because *coI* is the only transcript in *P. polycephalum* that is known to be processed by four different editing forms, its evolution provides a broad snapshot of the history and diversity of RNA editing.

MATERIALS AND METHODS

Cultures

Freeze-dried cultures of *S. flavogenita* (24714) were obtained from the American Type Culture Collection, and grown on half-strength cornmeal agar plates into full size plasmodia. Plates of *D. nigripes* plasmodia were obtained from Carolina Biologicals. Fruiting bodies of *A. cinerea* and *C. debaryanum* were a gift from the field collections of Steven Stephenson and Martin Schnittler.

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          1265                          1293
aci_pep   L  F  A  G   Y  Y  F  W  T  S
aci_rna   TTATTTGCTG GATACTATTT TTGGACATC
cde_pep   S  F  A  A   F  Y  Y  W  Y  S
cde_rna   TCATTTGCAG CATTTTATTA TTGGTATTC
dni_pep   A  F  A  A   F  Y  H  W  F  C
dni_rna   GCATTTGCTG CATTTTATCA CTGGTTTTG
sfl_pep   T  F  A  A   F  Y  H  W  F  C
sfl_rna   ACTTTTGCTG CATTCTATCA CTGGTTCTG
ppo_pep   A  F  A  A   F  Y  H  W  F  S
ppo_rna   GCTTTTGCTG CTTTTTATCA TTGGTTTTC
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FIGURE 5. Portion of *coI* alignment showing a U insertion at a homologous site with C insertions. Sequence names are as in Figure 3. The black box with a white letter indicates a U insertional editing site. The three gray boxes indicate sites of C insertional editing. The first nucleotide of the alignment (T/G/A) corresponds to position 1265 in GenBank AF239221.

Isolation of nucleic acids and reverse transcription

RNA and DNA were extracted from the slime mold plasmodia and fruiting bodies by use of Trizol reagent from Life Technologies. RNA was treated with DNase (Promega); DNA was treated with RNase A (Sigma). Nucleic acids were then extracted with phenol/chloroform, ethanol precipitated, and resuspended in T.E (10 mM Tris, pH 7.4, 0.1 mM EDTA). *S. flavogenita* and *D. nigripes* RNA was reverse transcribed with Superscript II reverse transcriptase from Life Technologies. *A. cinerea* and *C. debaryanum* RNA was reverse transcribed with SensiScript RT from Qiagen.

S. flavogenita coI cDNA amplification and sequencing

A nested polymerase chain reaction was performed on random hexamer primed cDNA, using primer coi141sm and coi510sm, followed by coi171sm and coi430sm. 3′ Anchor PCR (Dorit et al., 1993) was performed after reverse transcription with CUAUXR′T14, consisting of three nested PCRs: coi361st to CUAUXR′, coi371st to CUAUXR′, and coi381st to CUAUXR′. A second 3′ anchor PCR was performed by adding GTP tails to total RNA by yeast poly(A) polymerase as described (Martin & Keller, 1998) with 0.5 mM GTP. After reverse transcription with primer UXR′C12, the PCR consisted of 20 cycles with primer CUAUXR′ and primer coi551st, followed by 20 cycles with CUAUXR′ and primer coi561st. Two independent rounds of 5′ anchor PCR were performed as described (Landweber & Gilbert, 1993) by tailing hexamer primed reverse transcripts with terminal deoxytransferase from Promega. The first set of nested PCRs was with primers coi200st to UXRC12, followed by a PCR with coi190st to UXR. The second round was nested PCR starting with coi70st and UXRC12, followed by coi66st and UXR. All cDNA products were cloned using the TOPO TA cloning kit from Invitrogen. Plasmids were purified with the High Pure Plasmid Isolation Kit from Boehringer-Mannheim/Roche. Both strands of at least three plasmids in each region were subjected to automated sequencing in the facility at Princeton University.

S. flavogenita coI DNA amplification and sequencing

Two central segments of *S. flavogenita* DNA were amplified by PCR with primers coi191sm to coi410sm, and primer coi367st and primer coi600st. Both regions were directly sequenced in the automated facility at Princeton University. Three rounds of walking PCR were performed as described (Katz et al., 2000). In the 5′ direction, single-strand amplification was done with both coi200b and coi80stBIOTIN. These products were amplified by nested PCR with primer coi190st and UXRC12, followed by primers coi180st and UXRC12; and

primers coi74st and UXR, followed by coi72st and UXR, respectively. Products were cloned and sequenced as described above. In the 3′ direction, single-strand amplification was performed with primer coi551b. Nested PCR was performed with primer coi561st and UXR′C12, followed by primer coi581st and CUAUXR′. A single clone was obtained by this method, and the plasmid isolated and sequenced. Primer stem-ptR was designed at the 3′ most extreme of this clone sequence, then used in a 40 cycle PCR with primer coi551st. The PCR product was precipitated and subjected to direct sequencing with primer coi551st and stem-ptR.

D. nigripes, *A. cinerea*, and *C. debaryanum coI* amplification and sequencing

The *coI* sequence of the other myxomycetes was obtained in two PCRs of DNA and cDNA with primers coi181sm and coi420sm, and primers coi5′2asm and coi3′2asm. At least three clones each were sequenced as described above.

rDNA amplification and sequencing

The *S. flavogenita* SSU rRNA gene was amplified in a single PCR with primers SMNUR101 and SMNUR108 (Spiegel et al., 1995). The *D. nigripes* gene was amplified in two overlapping PCRs with primers SMNUR101 to SSUNS4; and SSU901sm to SSU2580sm. *A. cinerea* DNA was amplified with primers SSUNS5 and SMNUR108. *C. debaryanum* DNA was amplified with primers SSU101sm and SSU522R. At least three clones of each product were sequenced as described above.

Sequence alignment and tree construction

DNA and cDNA sequences were aligned, and consensus sequences were determined using the Seqlab interface of the Wisconsin Package Version 10.0 (GCG, 1999). *CoI* sequences were aligned with *P. polycephalum* sequences from GenBank (L14769 and L14779). Maximum likelihood and parsimony trees were constructed using PAUP* (Swofford, 1998) with nuclear SSU sequences obtained for the four myxomycetes, plus *P. polycephalum* (GenBank X13160), *D. discoideum* (X00134) and *Rhizomucor racemousus* (X54863).

Primer sequences

Primers mentioned are listed below. All nucleotides are in IUPAC standard notation, except bold “P” and bold “K” indicate synthetic pyrimidine and purine derivatives from Glen Research, and “BIO*” indicates a 5′ biotin.

coi141sm: GCWTTTCCWMGWTTAAA**P**AA**P**ATIWS**P**TTATGGTT
coi171sm: ATMGGWG**P**WGGAACWGGTTGGACWGTT
coi180st: CTAAAAATAGCTAAATCTACAG
coi181pds: GGWTGGACWGTTTATCCWCC
coi190st: TGAAGATACACCAGCTAGATG
coi191sm: CCACCATTAGCTTCTGTTGTTGGCC
coi200b: BIO*ACGCATATTTCTAACAGTACAG
coi200st: ACGCATATTTCTAACAGTACAG
coi3′2asm: GCATCWGGATAATCWGGRATACGRCG
coi361st: ACACCTTTATTATTTGTTTTAGG
coi367st: TTACTCTTTACCTTAGGTGGTCTTAGTGG
coi371st: TACCTTAGGTGGTCTTAGTGG
coi381st: TAGCAAATTCTGGTTTAGATG
coi391st: TTCTGGTTTAGATGTGCATTCCATGATAC
coi410sm: ACATAATGAAAATGAGCAACGAC
coi420pds: GCWCCCATDGATAAKACATAATG
coi430sm: GCAAAIA**P**WGCWCCCATWSWTAADAC
coi510sm: GCIA**K**WCCTAAKA**K**ATGCATWGG**K**AA**K**AAIGTAASATTWAC
coi5′2asm: GCATTAATTGGTGGSTTTGGWAAYTGG
coi551st: TTGTTAGCAAATGATTATCG
coi551stbiotin: BIO*TTGTTAGCAAATGATTATCG
coi561st: TACATTTCCTTTAACTGTTGC
coi581st: CTTATGTTTCTTCTTTAGATGC
coi600st: GTTTTAAATAGGCAAGTTTACGTG
coi66st: TCACCACAACCGGCTAATTCC
coi70st: GGGCATGTGCGGTAACAATGG
coi72st: GCACCGATACTAAATAAAAGATATAAAGTACC
coi74st: GATAATCCTAATAACCCAGCACC
coi80st: CTAATTCCATACGCATAATAAC
coi80stbiotin: BIO*GCTAATTCCATACGCATAATAAC
CUA-UXR′: CUACUACUACUACTCGAGAATT
CUA-UXR′C12: CUACUACUACUACTCGAGAATTCCCCCCCCCCCCD
CUA-UXR′T14: CUACUACUACUACTCGAGAATTTTTTTTTTTTTV
SMNUR 101: CTGGTTGATCCTGCCAGTAG
SMNUR 108: GTTACGACTTCTCCTTCCTC
SSU101sm: TCTGCGAACGGCTCCGCAAAC
SSU2580sm: ACGACTTCTCCTTCCTCTAGGCC
SSU522R: CCCAATGGGAACGTTGCGCG
SSU901sm: GGCTSGGGGTACCAATYACC
SSU-NS4: CTTCCGTCAATTCCTTTAAG
SSU-NS5: AACTTAAAGGAATTGACGGAAG
stem-ptR: TAAGTAAATGCAGTAACATTTG
UXR: CAUCAUCAUCAUCTCGAGAATT
UXRC12: CAUCAUCAUCAUCTCGAGAATTCCCCCCCCCCCCD

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