A widespread class of reverse transcriptase-related cellular genes

Eugene A. Gladyshev[†‡], Irina R. Arkhipova[†*]

[†]Josephine Bay Paul Center for Comparative Molecular Biology and Evolution, Marine Biological Laboratory, 7 MBL Street, Woods Hole, MA 02543, USA

[‡]Present address: Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA, USA

*To whom correspondence should be addressed. Email: iarkhipova@mbl.edu. Tel. (508) 289-7120. Fax (508) 457-4727.

Abstract

Reverse transcriptases (RTs) polymerize DNA on RNA templates. They fall into several structurally related but distinct classes, and form an assemblage of RT-like enzymes which, in addition to RTs, also includes certain viral RNA-dependent RNA polymerases (RdRP) polymerizing RNA on RNA templates. It is generally believed that most RT-like enzymes originate from retrotransposons or viruses and have no specific function in the host cell, with telomerases being the only notable exception. Here we report on the discovery and properties of a novel class of RT-related cellular genes collectively named *rvt*. We present evidence that *rvt* are not components of retrotransposons or viruses, but single-copy genes with a characteristic domain structure, may contain introns in evolutionarily conserved positions, occur in syntenic regions, and evolve under purifying selection. These genes can be found in all major taxonomic groups including protists, fungi, animals, plants, and even bacteria, although they exhibit patchy phylogenetic distribution in each kingdom. We also show that the RVT protein purified from one of its natural hosts, *Neurospora crassa*, exists in a multimeric form and has the ability to polymerize NTPs as well as dNTPs *in vitro*, with a strong preference for NTPs, using Mn^{2+} as a cofactor. The existence of a previously unknown class of single-copy RT-related genes calls for re-evaluation of the current views on evolution and functional roles of RNA-dependent polymerases in living cells.

\body

Introduction

DNA-dependent polymerases are essential for cellular function, as they mediate the flow of genetic information from DNA to RNA to proteins [1]. In contrast, RNA-dependent polymerases have long been associated with replication of selfish and parasitic genetic elements, such as viruses or transposons. While the discovery of reverse transcriptase (RT) challenged the concept of unidirectionality of the flow of genetic information, this reverse direction has been reserved for retroviruses, pararetroviruses (hepadna- and caulimoviruses), and other RT-containing multicopy entities such as non-LTR and LTR-retrotransposons, group II introns, retrons, and retroplasmids, as well as occasional retro(pseudo)genes [2-4]. Similarly, viral RNA-dependent RNA polymerases (RdRPs), enzymes structurally related to RTs, serve to replicate the genomes of viruses that use RNA as genetic material [5].These and certain other polymerases are unified by the architecture known as “right-hand”, composed of the three subdomains called fingers, palm, and thumb [6]. Like all polymerases, they use two-metal-ion catalysis for phosphoryl transfer reactions resulting in nucleotide addition.

In 1997, this diverse superfamily of enzymes was joined by the telomerase reverse transcriptase (TERT), a specialized RT which maintains the ends of eukaryotic linear chromosomes by addition of short G-rich repeated DNA sequences that are copied multiple times *via* reverse transcription of a specific region of the associated RNA template constituting part of the holoenzyme [7]. Telomerases are unique in being single-copy eukaryotic RT genes which do not represent a component of any mobile element or virus. It has been argued that, in early eukaryotic evolution, telomerases may have either descended from domesticated retrotransposons or have given rise to them [8,9]. In fact, TERTs were shown to be most closely related to RTs from *Penelope*-like retroelements (PLEs) [10-12]. However, TERT genes so far remain the only example of single-copy RT-related eukaryotic genes with a defined cellular function. In this study, we identify and characterize the second major group of RT-related cellular genes.

Results

Identification and characterization of *rvt* genes in bdelloid rotifers. We discovered *rvt* genes in the course of cloning and sequencing of telomeric regions from rotifers of the class Bdelloidea, small freshwater invertebrates which are best known for having evolved for millions of years apparently without males and meiosis; for their resistance to desiccation and ionizing radiation; and for their ability to acquire foreign genes from diverse sources [11,13-16]. During sequencing of fosmids from the genomic library of the bdelloid rotifer *Adineta vaga* (family Adinetidae), we found a member of a previously unrecognized group of RT-like genes, which we named *rvt* because it contains an identifiable *rvt* conserved domain (*rev*erse *t*ranscriptase; pfam00078:RVT_1). The *A. vaga rvt* did not fall into any of the known RT categories, such as retrons, retroplasmids, group II introns, telomerases, non-LTR retrotransposons, LTR retrotransposons, retroviruses, and pararetroviruses. Its single-copy status was established by Southern blot hybridization of genomic DNA and by exhaustive screening of the *A. vaga* genomic fosmid library [17]. In this library, we found a co-linear pair of *rvt*-containing fosmids with 4% overall divergence, consistent with the genome structure of bdelloid rotifers in which chromosomes occur as co-linear allelic pairs with overall divergence up to 6% [17,18]. The divergence between members of the *rvt* pair is less than 1%, either at the nucleotide level (13/2490 nt substitutions) or at the protein level (6/829 aa substitutions). Sequencing of *rvt*-containing *A. vaga* fosmids revealed that they are located in a subtelomeric region rich in telomeric repeats, telomere-associated *Athena* retrotransposons, and foreign genes of apparently bacterial or fungal origin (Supporting Information (SI) Fig. S1A).

Moreover, we found *rvt* in four other species of bdelloid rotifers (*Philodina roseola, P. acuticornis,* and *Macrotrachela quadricornifera* from the family Philodinidae, and *Habrotrocha rosa* from the family Habrotrochidae), using PCR and genomic library screens. On a contig from the *P. roseola* genomic library, *rvt* is located between two genes of apparently bacterial origin (SI Fig. S1B). There are two distinct lineages of *rvt* genes in bdelloids, A and B, which could originate from two independent acquisition events (SI Fig. S1C). However, while the *A. vaga rvtA* has a slightly higher GC-content than neighboring genes, there is no detectable difference in GC content and codon usage between *P. roseola rvtB* and adjacent genes, indicating that if it was also acquired by lateral transfer, it took place a sufficiently long time ago for the differences to have ameliorated.

Structure and distribution of *rvt* in sequenced genomes. Comparison of rotifer *rvt* genes with their homologs in other kingdoms reveals their highly conserved overall structure, which deviates significantly from all presently known RT types (Fig. 1). A typical *rvt* ORF is 800-1000 aa in length. The core RT domain, which contains RT motifs 1 through E (fingers and palm), plus the thumb subdomain, is framed at the N- and C-termini by well-conserved 300- and 200-aa extensions, respectively, which reveal no homology to known motifs other than the coiled-coil motif at the very N-terminus. The two neighboring aspartates in the core motif C, which constitute part of the D,DD catalytic triad, are typically preceded by a non-canonical histidine residue. In agreement with the single-copy nature of *rvt* genes, their core RT

domain is not associated with any domains resembling known endonucleases or integrases, which are usually responsible for intragenomic mobility. A distinctive structural feature of *rvt* genes is a large insertion loop separating motifs 1-2 from the rest of the core RT (70-100 aa, and up to additional 70 aa in the Mo lineage, see below; Fig. 1; SI Fig. S2), which is enriched in acidic Asp and Glu residues and confers a net negative charge to the molecule, with an average isoelectric point of 5.5.

In BLASTP searches, *rvt* genes retrieve each other, but not other types of RTs (with occasional low-significance hits to non-LTR retrotransposons and group II introns). In a CD(conserved domain)-search, many of them fit the profile RT_like_1 (cd01709: "an RT gene usually indicative of a mobile element such as a retrotransposon or retrovirus") composed of 14 fungal sequences, although this profile lacks core RT domains 1 and 2 as they are separated by the large loop, and is rarely retrieved by non-fungal *rvt* genes, which yield matches only at the next hierarchical level (cd00304: RT_like superfamily).

Although the distribution of *rvt* genes is rather patchy, they occur in all eukaryotic kingdoms: protists, fungi, animals, and plants. These genes are present in a highly diverse set of species with sequenced genomes: 60 fungi (not only euascomycetes and basidiomycetes, but also chytrids and microsporidia, the most basal fungal taxa); the moss *Physcomitrella patens*; six stramenopiles (heterokonts), including the genera *Phytophthora*, *Saprolegnia*, and *Pythium*; and a bacterium (Fig. 2; SI Table S1). In EST databases, there are also two homologous fragments from an arthropod (Arctic springtail *Onychiurus arcticus*), which however exhibit some similarity to *rvt* from a microsporidian parasitizing on mosquitoes (*Vavraia culicis*). Of special interest is the existence of *rvt* genes in the sequenced genome of the filamentous gliding bacterium *Herpetosiphon aurantiacus* (Chloroflexi) and two uncultured environmental bacteria. Finding the same type of RT in both prokaryotes and eukaryotes is so far unprecedented. However, despite its basal position, this RT may have originated from a rare eukaryote-to prokaryote horizontal transfer, as *rvt* genes occasionally exhibit phylogenetic discordance (*e.g*. one of the bdelloid lineages groups with basidiomycetes; *rvt* from the moss *Physcomitrella* forms a clade with *Tuber*, a basal ascomycete; and *rvt* from the basidiomycete *Ustilago* is found within an ascomycete lineage) (Fig. 2).

Phylogenetic analysis of *rvt* genes reveals that they underwent duplications early in ascomycete evolution, as well as sporadic loss of members from each duplicated lineage. The phylogram in Fig. 2 shows an early duplication event leading to formation of lineages Mo and Nc, and an even earlier duplication leading to formation of lineages Pa and Ts (each lineage is denoted after the species which carries only this lineage: Nc-*Neurospora crassa*, Mo-*Magnaporthe oryzae*, Ts-*Talaromyces stipitatus*, Pa-*Podospora anserina*, Lm-*Leptosphaeria maculans*). In some ascomycetes, such as *Phaeosphaeria nodorum* (Dothideomycetes) or *Aspergillus spp.* (Eurotiomycetes), representatives of three or four lineages are present simultaneously, which indicates early divergence and subsequent loss of different lineage members from certain species, and may also indicate partial redundancy of *rvt* function in different lineages. Members of the minor lineage L are not likely to possess catalytic activity, since they lack one of the conserved aspartates in the D,DD triad, and hence form a very long branch. We also observed several recent *rvt* losses: for instance, in *Epichloe festuca* and *Coprinopsis cinerea*, only a ~100-aa fragment can be recognized, and in *Neosartorya fischeri*, the Ts lineage appears intact, while the Pa lineage is represented by a fragment with a large internal deletion spanning nearly 700 aa. In addition, in six cases one of the lineages contains in-frame stop codons and/or frameshifts, while another appears intact. At the same time, *rvt* is absent from the genomes of 15 sequenced euascomycetes, and is not found in any of the 35 sequenced yeast genomes. Although incomplete coverage may occasionally account for such absence, the lack of *rvt* in three *Arthroderma spp*., four *Trichophyton spp*., and three *Trichoderma spp*. is more likely to indicate secondary loss.

Synteny in *rvt* genomic environments. In each host species, *rvt* is present either as a single-copy gene or as a 2-3-member gene family. If these genes are not mobile elements, and two or three *rvt* copies are

found in related genomes, synteny in their genomic environment in related species would clearly indicate that such copies were not derived from recent retrotransposition, but from ancient duplication. We investigated whether *rvt* genes in sequenced fungal genomes are located in chromosomal regions exhibiting appreciable degrees of synteny. Analysis of genomic contigs carrying the most divergent members of the Ts lineage clearly shows that they are located in syntenic regions (SI Fig. S3A). Although several inversions occurred within syntenic blocks, the overall synteny can be traced prior to separation of the orders Eurotiales (*Aspergillus, Penicillum*) and Onygenales (*Uncinocarpus, Paracoccidioides*), dated between 150 and 400 Mya depending on molecular clock calibration [19]. Members of other *rvt* lineages exhibit similar degrees of synteny (Fig. 2), which is typically observed within a class, although occasionally cannot be traced to that level due to insufficient contig length. Preservation of synteny for tens of millions of years is typical of nuclear genes and is not characteristic of mobile genetic elements.

Selective forces acting on *rvt* genes. If *rvt* genes are not mobile elements, but have evolved to perform a certain function in the host, orthologous copies should exhibit evidence of selective pressure which acts to preserve that function. We asked whether *rvt* genes in related species evolve under purifying selection. To this end, we compared the rates of non-synonymous and synonymous amino acid substitutions in pairs of orthologous *rvt* copies from fungal genomes. In each case, we observed four- to ten-fold excess of synonymous over non-synonymous substitutions, which is strongly indicative of purifying selection (SI Fig. S3B). In bdelloid rotifers, *rvt* genes also evolve under purifying selection, as evidenced by comparison of *rvtB* in *P. roseola* and *M. quadricornifera* (SI Fig. S3B). The same pattern holds for all four species in the genus *Phytophthora* (Stramenopiles, or Oomycetes). Interestingly, signatures of selection can be revealed even in comparisons between recent duplications: in the basidiomycete *Fomitiporia mediterranea*, three adjacent *rvt* copies display a fivefold excess of synonymous substitutions. Thus, *rvt* genes are under strong selective pressure in several unrelated groups of fungi, protists, and animals.

Intron distribution. Introns in nuclear genes are widespread, while in retroelements they are highly unusual, since the corresponding cDNA is synthesized on processed mRNA templates and is not expected to retain introns (but see [10]). Preservation of the exon-intron structure over evolutionarily long periods of time served as evidence for lack of retromobility of TERT genes, many of which contain introns [20]. We therefore examined the patterns of intron occurrence in *rvt* genes. While the coding regions of *rvt* from fungal lineages Lm and Ts do not contain introns, in lineages Pa, Mo and Nc there are many copies that do (triangles in Fig. 2). Conserved introns can also be found in noncoding regions (see below), although these are more difficult to detect in the absence of adequate transcriptome coverage. Moreover, while a few introns appear to have arrived late, many intron positions are shared between different genera, indicating that these introns have been acquired relatively early in evolution (Fig. 2). Although alternative explanations, such as independent intron insertion into specific sites, cannot be ruled out, it appears likely that the presence of shared intron positions reflects common ancestry, as it is typically accompanied by synteny in *rvt* genomic environments.

Transcription patterns. The first glimpse of *rvt* expression patterns may be obtained from BLAST searches of the available EST databases. EST analysis indicates that *rvt* genes are normally expressed at relatively low levels: transcripts can be detected, on average, with a frequency of 1-2 per 10-15,000 sequenced EST tags, with a few exceptions (SI Table S1). Our RT-PCR experiments with *N. crassa* (mycelium) and *A. vaga* (whole animals) RNA show that in both species *rvt* is weakly transcribed, and 5'-RACE analysis demonstrates that the 5'-untranslated region (UTR) of the *N. crassa rvt* gene contains a 62-bp intron, which is conserved in *N. tetrasperma*, *N. discreta,* and *Sordaria macrospora* (Fig. S4A). Remarkably, inspection of transcriptional profiling data shows that in the *N. crassa* strain mutant for the gene encoding a global transcriptional regulator CPC1 (cross pathway control-1)*,* a yeast *GCN4* ortholog, the level of *rvt* transcription undergoes a 47-fold increase under conditions of amino acid starvation

induced by 3-aminotriazole (3-AT), an inhibitor of histidine biosynthesis [21]. This increase is higher than that for any other gene out of 10,526 *N. crassa* genes. We verified this result by RT-PCR, and showed that addition of histidine restores normal expression levels (Fig. 3A). Thus, *rvt* expression in *N. crassa* appears to be under tight control, and under certain stress conditions its levels may rise dramatically.

Importantly, we found that *rvt* expression is strongly induced in the wild-type strain when protein synthesis is inhibited not only by the lack of histidine, but by other means as well. Addition of antibiotics blasticidin S or cycloheximide to the exponentially growing *N. crassa* mycelium increases *rvt* expression by several orders of magnitude (Fig. 3A). These two antibiotics affect protein synthesis by different mechanisms, *i.e*. by blocking peptide bond formation and translocation steps, respectively [22,23]. Remarkably, the concentration of blasticidin needed for full induction (0.1µg/ml) is at least an order of magnitude lower than that normally used to suppress protein synthesis (5-50 µg/ml), and does not strongly affect the growth rate, as does cycloheximide (Fig. 3B). Moreover, the increase in *rvt* transcription is paralleled by a similar increase in levels of the *rvt*-encoded protein (see below). We also find that several other genes, including an AAA+ ATPase, are strongly induced by addition of blasticidin at low concentration (SI Fig. S4B).

Protein purification and *in vitro* activity assays. For initial characterization of RVT activity *in vitro*, we first sought to overexpress the full-length 890-aa NcRVT protein, tagged with an N-terminal 6xHis affinity tag, by introducing it into the *rvt* knockout *N. crassa* strain using homologous transformation (see SI Methods). This strain displays no visible phenotype under laboratory conditions. Although the 6xHis tag did not bind to the Ni-affinity column as expected, it allowed us to track expression of the tagged protein in *N. crassa* extracts on SDS-polyacrylamide gels by Western blotting with the His-tag-specific antibody (SI Fig. S4E and Fig. 3E), and to adjust conditions under which it could be purified by ammonium sulfate fractionation and sucrose gradient centrifugation. Major improvement was achieved when we found that expression of the NcRVT protein is induced by several orders of magnitude under conditions which inhibit protein synthesis (Fig. 3A). Upon induction with 0.1 µg/ml blasticidin, untagged RVT protein could be purified to near homogeneity, as judged by SDS-PAGE which reveals a major 102-kDa band (Fig. 3C). In sucrose gradients, however, NcRVT sediments faster than all commercially available high molecular weight markers, and extrapolation of the calibration curve indicates that it likely forms a 1-MDa decameric complex (SI Fig. S5). The identity of untagged NcRVT protein was confirmed by mass-spectrometry following tryptic digestion (SI Fig. S6).

We next sought to confirm the ability of NcRVT to act as a polymerase *in vitro*. The purified protein has the capacity to polymerize both NTPs and dNTPs, with a strong preference for NTPs, using Mn^{2+} as a cofactor. Purified NcRVT was first incubated with α-^{32}P-dCTP, and then chased with an excess of cold NTPs or dNTPs (Fig. 3D). Addition of pyrimidine nucleotides typically results in longer extension products. Addition of Mg^{2+} instead of Mn^{2+} resulted in a significant decrease in length and intensity of extended products (SI Fig. 4C). Importantly, polymerization is completely abolished when one of the catalytic aspartates is replaced by alanine in the His-tagged version (Fig. 3E). Substitution of α-^{32}P-dCTP with γ-^{32}P-ATP does not result in appearance of visible extension products, indicating that *de novo* initiation is not occurring *in vitro* (SI Fig. 4C). All extension products have a minimum length of *ca*. 10 nt, suggesting that α-^{32}P-CTP is being added to pre-bound primers present in the purified NcRVT (SI Fig. 4D-E). Addition of various exogenous primer/template combinations did not result in primer extension products, although endogenous RNA primers, represented by either natural 3'-termini or cleavage fragments of abundant cellular RNAs or short oligomers, were readily extended by the terminal nucleotidyltransferase activity upon incubation with an NTP and Mn^{2+}, resulting in addition of up to 200-nt homopolymeric tails as verified by cloning and sequencing.

Relationship of *rvt* genes to other RT sequences. The ability of RVT to add NTPs and, to a lesser extent, dNTPs to 3'-OH termini places it apart from conventional RTs and closer to TERTs, which are known to exhibit RdRP and template-independent terminal deoxynucleotidyltransferase (TdT) activity in addition to RT activity [24,25]. The emergence of a novel type of RT-related genes raises questions about their relationship to other RT-like proteins. To clarify this relationship, we compiled an extended RT dataset (see [10]) including representatives of every known group of RT-like proteins, including retrons, retroplasmids, group II introns, diversity-generating retroelements (DGR), retroviruses, pararetroviruses (hepadna- and caulimoviruses), LTR retrotransposons (gypsy, copia, BEL, DIRS), non-LTR retrotransposons, *Penelope*-like elements (PLE), telomerases (TERT), and *rvt* genes. Using profile-profile searches and structure-based alignments, the core RT region was extended in both directions to span the entire *ca*. 500-aa minimal functional RT, such as that found in retrons (SI Fig. S7). The *rvt* thumb domain aligns most readily with that of RT-derived *Prp8* genes [26] and non-LTR retrotransposons (SI Fig. S2), ending with a highly conserved GGLG motif, which may serve as a flexible hinge connecting the thumb domain and a C-terminal extension. Similarities in secondary structure broadly subdivide RT-related sequences into four supergroups: a rather loose supergroup includes prokaryotic RTs (P), another unites virus-like entities such as LTR retrotransposons, retroviruses, and pararetroviruses (V), the third one consists of PLEs and TERTs (T), and the last one (L) shows affiliation between non-LTR retrotransposons and *rvt* genes, despite the lack of a conserved motif 2a in the latter.

Both traditional and phylogenetic network analysis (Fig. 4A,B), which visualizes uncertainties from conflicting phylogenetic signals, largely agree with these subdivisions, which can be further reinforced by additional synapomorphies, such as the presence of RNase H domain in virus-derived RTs, or the presence of similarly structured N- or C-terminal extensions in other supergroups. While all *rvt* genes are always grouped together with 100% support, other RT classes, such as non-LTR and LTR retrotransposons, are much more diverse. Overall, these findings demonstrate an ancient origin of *rvt* genes and point at independent evolutionary origins of different classes of retrotransposons, which were likely formed by fusion of ancestral RT domains with different types of endonucleases during early eukaryotic evolution.

Discussion

Forty years after the discovery of RTs in vertebrate retroviruses [2,3], these enzymes have expanded into a large and diverse megafamily, members of which are usually assigned to various selfish genetic elements such as retrotransposons, retroviruses or pararetroviruses. Also included in the RT-like sequence cluster are the RdRPs of dsRNA and positive-strand ssRNA viruses of the picorna-like superfamily [27]. In all of the above cases, RNA-dependent synthesis serves the sole purpose of replication of selfish genetic elements of transposable or viral nature. A notable exception to this rule is telomerase, a specialized RT performing RNA-templated DNA synthesis at eukaryotic chromosome ends [28,29].

The newest addition to the RT-like megafamily described herein, the *rvt* genes, bear a certain degree of resemblance to telomerases in being not multicopy, but single-copy genes, which evolve under purifying selection and do not exhibit varying localization in host genomes. It may therefore be argued that the role of RNA-dependent synthesis in eukaryotic cells is not restricted to maintenance of chromosome ends. Furthermore, *rvt* genes, like TERTs, have acquired additional domains which do not bear resemblance to endonuclease domains typically associated with core RT domains in retrotransposable elements, and do not confer retromobility, but could be involved in primer, template, or protein-protein interactions.

One cannot help but wonder at a highly unusual pattern of *rvt* phylogenetic occurrence, which is not restricted to any specific domain of life, but nevertheless exhibits patchy distribution within each of the

major kingdoms. So far, the most prominent *rvt*-carrying taxonomic group is the fungal kingdom, including 46 out of 65 sequenced euasomycetes, 8 out of 32 basidiomycetes, and 1 out of 3 chytrids. This pattern may, to a certain extent, reflect the bias in genome sequencing: ascomycete genomes are among the easiest to sequence and assemble, while basidiomycete genomes have not yet reached this level of coverage. The same logic may also be applied to other compact sequenced genomes, such as stramenopiles, half of which do carry *rvt* genes. However, several taxonomic groups exhibit clear-cut cases of *rvt* loss.

One of the most puzzling observations is the apparently universal occurrence of *rvt* genes in bdelloid rotifers: out of five bdelloid species investigated, at least one *rvt* lineage could be detected in each of them. While *rvt* genes from lineage B are clearly related to *rvt* from basidiomycetes, those from lineage A appear more similar to oomycete *rvt*, pointing at independent introduction events. The fact that members of each lineage are found in genomic regions rich in genes of bacterial and fungal origin argues in favor of *rvt* introduction into bdelloids by horizontal transfers, which may have taken place prior to diversification of the major bdelloid families. So far, we were unable to detect *rvt* in partially sequenced genomes of monogonont rotifers, which do not undergo frequent cycles of desiccation and rehydration and are apparently not subject to massive horizontal gene transfers. It is also unlikely that *rvt* genes exist in any of the chordate genomes, which have been extensively sampled (66 total, mostly mammalian). However, it is quite possible that additional *rvt* genes will be found in other invertebrate and plant genomes, which still remain under-sampled and under-assembled.

Preservation of synteny in the environment of fungal *rvt* lineages can be traced as far back as the taxonomic rank of a class (e.g. Eurotiomycetes), implying early divergence of *rvt* lineages by duplication. Intron distribution largely correlates with synteny. While no intron position is conserved in all *rvt* lineages, several deep-branching lineages do share introns between all representatives. In bdelloid rotifers, intron insertion into lineage B occurred prior to divergence of the family Philodinidae, and both lineages were apparently introduced into the common bdelloid ancestor prior to divergence of the major bdelloid families, which took place tens of millions of years ago [13].

We also show that the purified RVT protein from *N. crassa* exerts the ability to polymerize NTPs, proving that it is a functional representative of the polymerase family. This activity depends on the presence of the highly conserved catalytic aspartate, and could not be detected in the *rvt* knockout *N. crassa* strain, ruling out participation of an endogenous RdRP. Nevertheless, polymerization was observed only in the presence of Mn^{2+}, which has less stringent coordination requirements than Mg^{2+} and allows use of suboptimal substrates and extra conformational flexibility [30]. However, *rvt* sequence is much more similar to RTs than to viral RdRPs, and its conserved motifs A and B do not carry residues that are chemically similar and positionally equivalent to those responsible for choice of NTP over dNTP *via* formation of hydrogen bonds with the 2'- and 3'- ribose oxygens in RdRP [31]. If *rvt* also exhibits preference for NTPs *in vivo*, the basis for such preference has to be different from that employed by viral RdRPs, although other RTs can switch preferences rather easily [32]. We hypothesize that the enzyme does not perform template-dependent synthesis indiscriminately, and would likely require additional processing and/or interaction with cofactors for full activity. In particular, it may have to undergo dissociation from the multimeric state and/or conformational/structural changes leading to displacement or removal of the loop region. *In vitro* utilization of various endogenous RNA primers, including high-abundance host RNAs and shorter RNA oligomers, together with the inability to extend exogenously added primers and primer/template combinations, indicates that these RNAs could become captured by the enzyme either within the cell or during isolation, although it is not clear whether they represent natural primers and templates.

Although *rvt* is not an essential gene, its evolutionary conservation and strong signatures of purifying selection across a variety of species strongly argue in favor of its involvement in cellular processes, which are yet to be identified. Its expression appears to be tightly linked to protein metabolism: it is greatly enhanced upon inhibition of protein synthesis in a variety of ways, such as amino acid starvation and interference with peptide bond formation or ribosome translocation by antibiotic addition. Interestingly, we identified another *N. crassa* gene, AAA+ ATPase, which is strongly induced under the same conditions. These chaperone-like ATPases are associated with the assembly, operation, and disassembly of protein complexes. It remains to be seen whether these and other proteins functionally interact with *rvt*. Future studies will explore possible involvement of *rvt* genes in stress response, repair of radiation- and desiccation-induced DNA damage, and genome defense.

The unique phylogenetic position of *rvt* genes and their apparent relatedness to LINE-like RTs bears relevance to the long-standing question whether cellular RTs, such as telomerases or *rvt*, could have originated from domesticated retrotransposons, or represent originally cellular genes which could have given rise to RT-containing selfish genetic elements. In prokaryotes, single-copy DGR RTs can assist phages in tropism switching, conferring selective advantage in the arms race with a bacterial host [33]; however, their function in bacterial genomes still remains a mystery. Telomerases are so far the only example of an essential RNA-dependent DNA polymerase gene in eukaryotes, as *Prp8* genes have lost the catalytic aspartate responsible for polymerization. Our findings indicate that RT-related genes are a lot more common than previously thought and may have evolved different functions *via* acquisition of various N- and C-terminal extensions, and that retrotransposons could have originated several times in early evolution through association with different types of endonuclease domains that confer intragenomic mobility.

Materials and Methods

Library screening, subcloning, and sequencing were done as described in [11,15]. Detailed procedures for protein purification, PCR, activity assays, and bioinformatic analyses are described in SI Text.

Acknowledgements

We thank M. Meselson for providing access to *A. vaga* and *P. roseola* genomic libraries and stimulating discussions, D. Mark Welch for sharing unpublished rotifer sequences, and three anonymous reviewers for constructive comments. This work was supported by NSF grant MCB-0821956 to I.A.

References

1. Crick F (1970). Central dogma of molecular biology. *Nature* 227: 561–563.
2. Baltimore D (1970) RNA-dependent DNA polymerase in virions of RNA tumor viruses. *Nature* 226:1209-1211.
3. Temin HM, Mizutani S (1970) RNA-dependent DNA polymerase in virions of Rous sarcoma virus. *Nature* 226:1211–1213.
4. Eickbush TH, Malik H (2002) Origins and evolution of retrotransposons. In: Craig NL, Craigie R, Gellert M, Lambowitz AM, eds., Mobile DNA II. Washington DC: ASM Press.
5. Ng KK, Arnold JJ, Cameron CE (2008) Structure-function relationships among RNA-dependent RNA polymerases. *Curr Top Microbiol Immunol* 320:137-56.
6. Steitz TA (1999). DNA polymerases: structural diversity and common mechanisms. *J Biol Chem*. 274:17395-17398.
7. Lingner J *et al*. (1997) Reverse transcriptase motifs in the catalytic subunit of telomerase. *Science* 276:561-567.

8. Eickbush TH (1997) Telomerase and retrotransposons: which came first? *Science* 277:911.
9. Nakamura TM, Cech TR (1998) Reversing time: origin of telomerase. *Cell* 92:587-90.
10. Arkhipova IR, Pyatkov KI, Meselson M, Evgen'ev MB (2003) Retroelements containing introns in diverse invertebrate taxa. *Nat Genet* 33:123-124.
11. Gladyshev EA, Arkhipova IR (2007) Telomere-associated endonuclease-deficient *Penelope*-like retroelements in diverse eukaryotes. *Proc. Natl. Acad. Sci. USA* 104: 9352-9357.
12. Chang GS *et al*. (2008) Phylogenetic profiles reveal evolutionary relationships within the "twilight zone" of sequence similarity. *Proc Natl Acad Sci USA* 105:13474-13479.
13. Mark Welch D, Meselson M (2000) Evidence for the evolution of bdelloid rotifers without sexual reproduction or genetic exchange. *Science* 288:1211-1215.
14. Gladyshev E, Meselson M (2008) Extreme resistance of bdelloid rotifers to ionizing radiation. *Proc Natl Acad Sci USA* 105:5139-5144.
15. Gladyshev EA, Meselson M, Arkhipova IR (2008) Massive horizontal gene transfer in bdelloid rotifers. *Science* 320:1210-1213.
16. Gladyshev EA, Arkhipova IR (2010) Genome structure of bdelloid rotifers: shaped by asexuality or desiccation? *J Hered* 101:S85-93.
17. Hur JH, Van Doninck K, Mandigo ML, Meselson M (2009) Degenerate tetraploidy was established before bdelloid rotifer families diverged. *Mol Biol Evol* 26:375-383.
18. Mark Welch DB, Mark Welch JL, Meselson M (2008) Evidence for degenerate tetraploidy in bdelloid rotifers. *Proc Natl Acad Sci USA* 105:5145-5149.
19. Taylor JW, Berbee ML (2006) Dating divergences in the Fungal Tree of Life: review and new analyses. *Mycologia* 98:838-849.
20. Nakamura TM *et al*. (1997) Telomerase catalytic subunit homologs from fission yeast and human. *Science* 277:955-959.
21. Tian C, Kasuga T, Sachs MS, Glass NL (2007) Transcriptional profiling of cross pathway control in *Neurospora crassa* and comparative analysis of the Gcn4 and CPC1 regulons. *Eukaryot Cell* 6:1018-1029.
22. Hansen JL, Moore PB, Steitz TA (2003) Structures of five antibiotics bound at the peptidyl transferase center of the large ribosomal subunit. *J Mol Biol* 330:1061-1075.
23. Schneider-Poetsch T *et al*. (2010) Inhibition of eukaryotic translation elongation by cycloheximide and lactimidomycin. *Nat Chem Biol* 6:209-217.
24. Lue NF *et al*. (2005) Telomerase can act as a template- and RNA-independent terminal transferase. *Proc Natl Acad Sci USA* 102:9778-9783.
25. Maida Y *et al*. (2009) An RNA-dependent RNA polymerase formed by TERT and the RMRP RNA. *Nature* 461:230-235.
26. Dlakić M, Mushegian A (2011) Prp8, the pivotal protein of the spliceosomal catalytic center, evolved from a retroelement-encoded reverse transcriptase. *RNA* 17:799-808.
27. Koonin EV, Wolf YI, Nagasaki K, Dolja VV (2008) The Big Bang of picorna-like virus evolution antedates the radiation of eukaryotic supergroups. *Nat Rev Microbiol* 6:925-939.
28. Autexier C, Lue NF (2006) The structure and function of telomerase reverse transcriptase. *Annu Rev Biochem* 75:493-517.
29. Wyatt HD, West SC, Beattie TL (2010) InTERTpreting telomerase structure and function. *Nucleic Acids Res* 38:5609-5622.
30. Yang W, Lee JY, Nowotny M (2006) Making and breaking nucleic acids: two-$Mg2^{+}$-ion catalysis and substrate specificity. *Mol Cell* 22:5-13.
31. Gong P, Peersen OB (2010) Structural basis for active site closure by the poliovirus RNA-dependent RNA polymerase. *Proc Natl Acad Sci USA* 107:22505-22510.
32. Gao G, Orlova M, Georgiadis MM, Hendrickson WA, Goff SP (1997) Conferring RNA polymerase activity to a DNA polymerase: a single residue in reverse transcriptase controls substrate selection. *Proc Natl Acad Sci USA* 94:407-411.
33. Medhekar B, Miller JF. (2007) Diversity-generating retroelements. *Curr Opin Microbiol* 10:388-395.

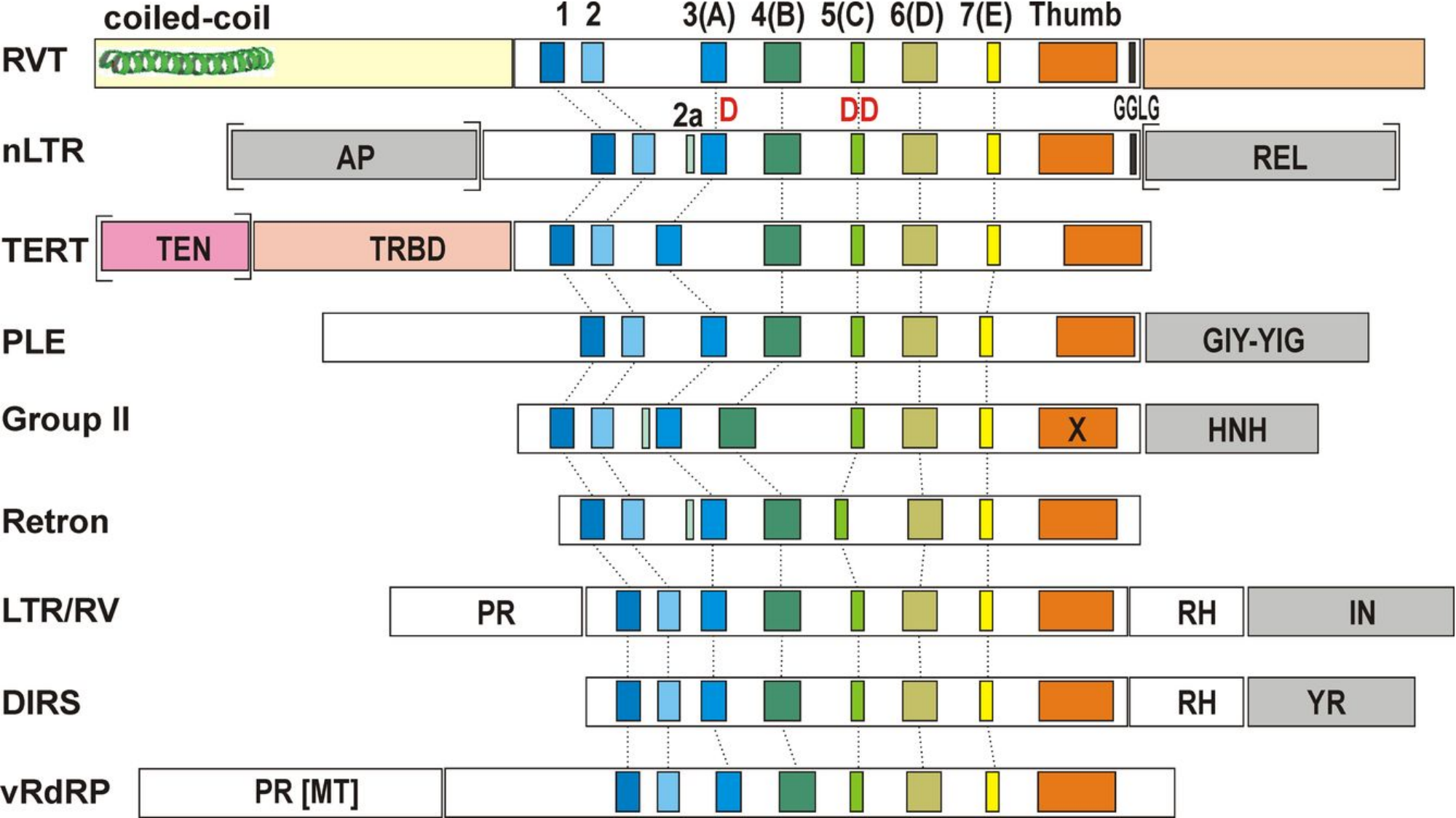
Figure Legends

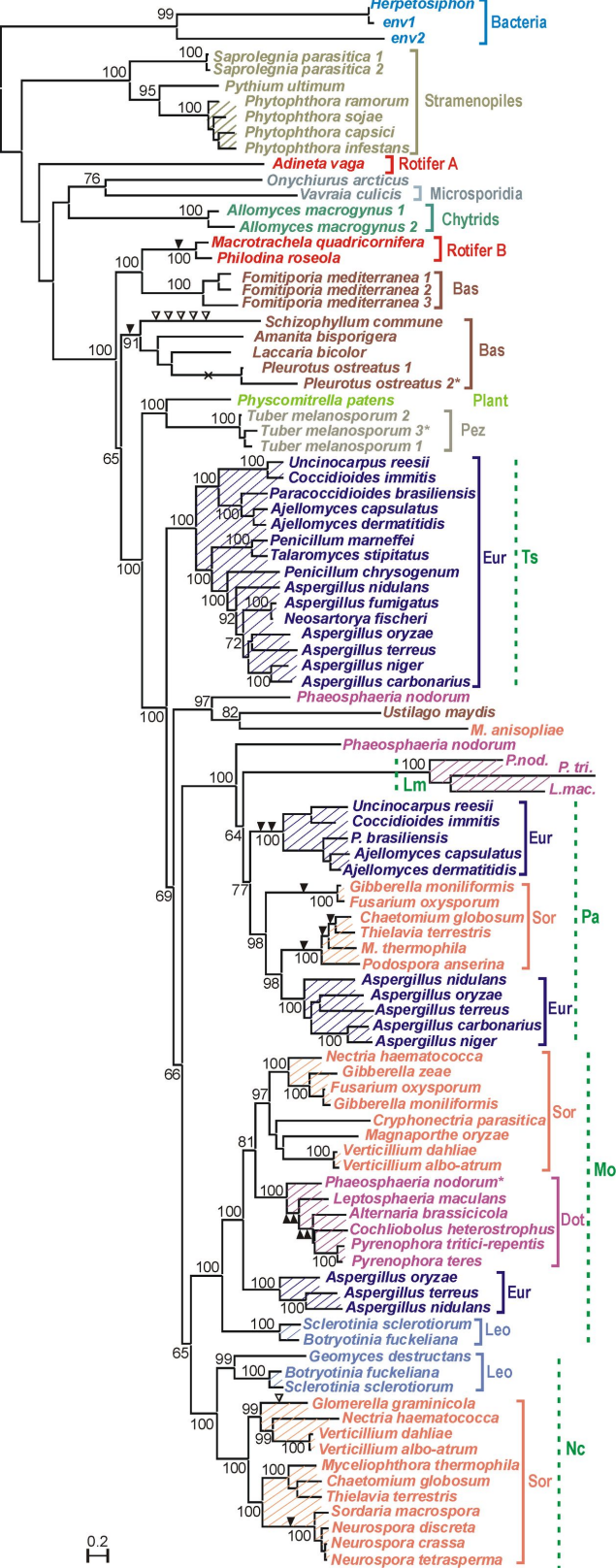
Fig. 1. Domain structure of *rvt* and representative members of the RT-like sequence cluster (NCBI-CDD cl02808 superfamily). Shown are the conserved core RT motifs 1 through 7, and the adjacent N- and C-terminal domains. Associated endonuclease domains of various types (AP, REL, GIY-YIG, HNH, IN, YR) are shown in gray. Other domains are abbreviated as follows: TEN, telomerase essential N-terminal; TRBD, telomerase RNA binding domain; PR, protease, RH, RNase H; MT, methyltransferase; X, maturase. Domains indicated by square brackets may or may not be present (*e.g*. non-LTR elements may contain either AP or REL endonuclease, or both). Also shown are the positions of the catalytic D,DD triad and the GGLG motif shared between *rvt* and early-branching non-LTR retrotransposons. Not shown are RTs of pararetroviruses and *copia*-like LTR retrotransposons.

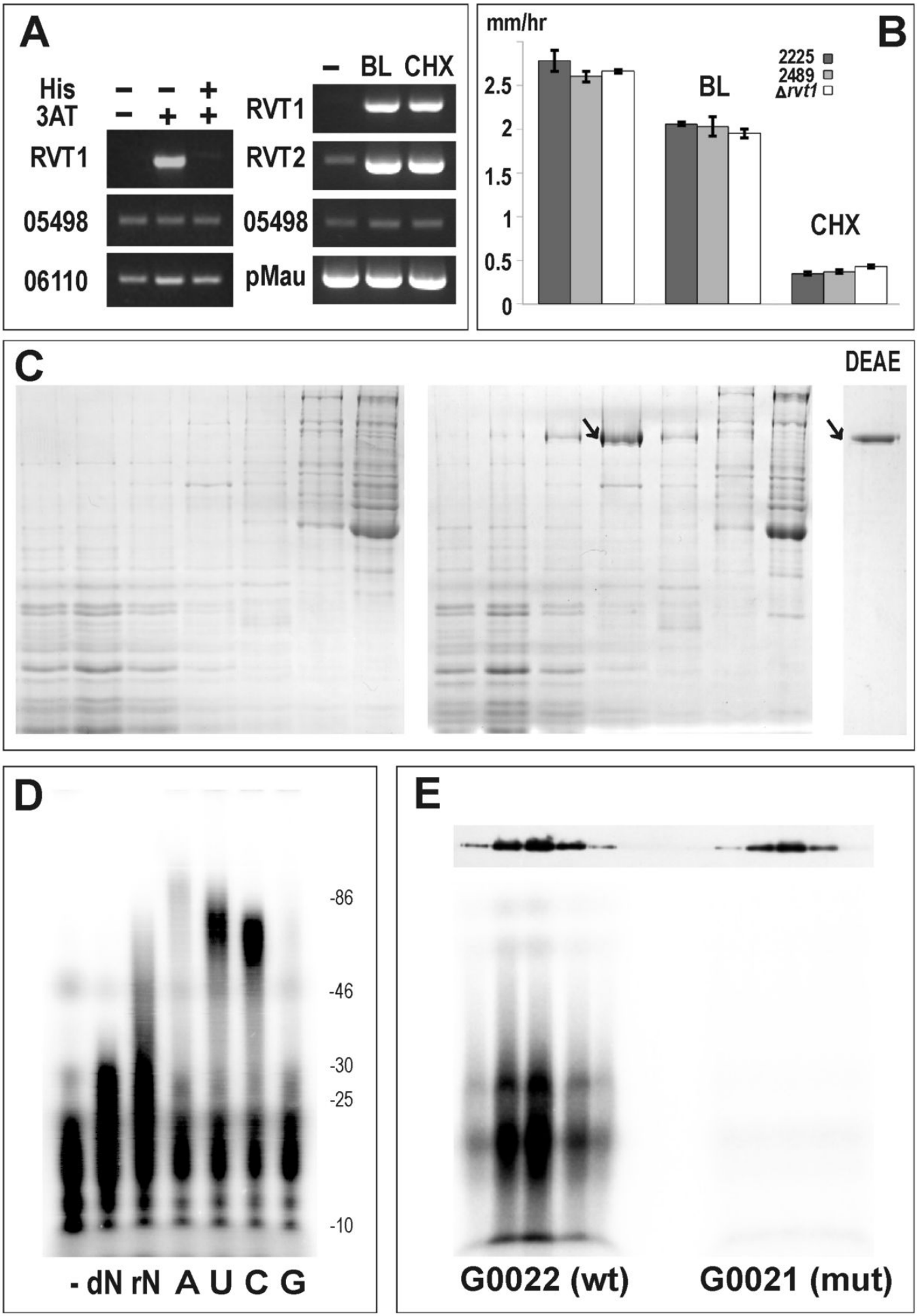
Fig. 2. A maximum-likelihood phylogram of 100 representative *rvt* protein-coding sequences (SI Dataset). Bootstrap support values exceeding 60% are indicated at the nodes. Cross-hatching indicates synteny in *rvt* genomic environments. Shared intron positions are denoted by filled triangles; unique positions, by open triangles; putative intron loss, by X. Asterisks denote copies with several frameshifts or in-frame stop codons. Color-coded taxonomic groups are as follows: Sor, Sordariomycetes; Eur, Eurotiomycetes; Dot, Dothideomycetes; Leo, Leotiomycetes; Pez, Pezizomycetes; Bas, Basidiomycetes. Nc, Mo, Lm, Pa and Ts denote *rvt* lineages (see text).

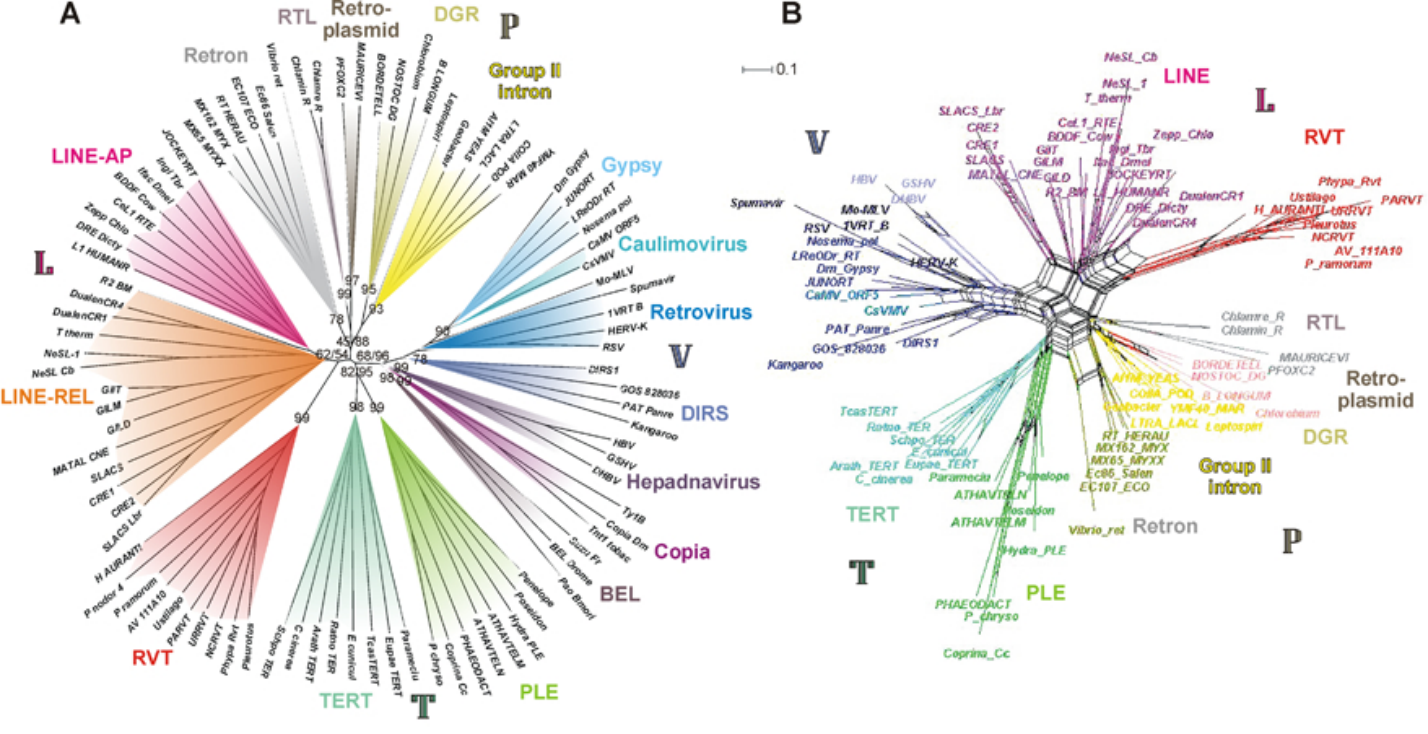
Fig. 3. Properties of *N. crassa rvt*. (**A**) Semi-quantitative RT-PCR showing response of *rvt* to 3-AT in the *cpc-1* mutant (FGSC#4264) with and without histidine addition (left), and response of *rvt* in the wild-type Mauriceville strain (FGSC#2225) to blasticidin (BL) and cycloheximide (CHX) (right). Expression from genes NCU5498, NCU06110, and the Mauriceville plasmid (pMau) was monitored as a control. (**B**) Effect of blasticidin and cycloheximide on mycelial growth rates for Δ*rvt* and two wild-type strains. (**C**) Sucrose gradient fractionation and DEAE chromatography purification of NcRVT from blasticidin-induced (right panel) and non-induced (left panel) Mauriceville strain. The position of the 102-kDa NcRVT protein in stained SDS-PAGE is indicated by an arrow. Odd-numbered fractions 17 through 29 are shown from left to right in each panel; numbering begins from bottom. The rightmost lane depicts the eluate from the DEAE column, which contains pure NcRVT protein. (**D**) Nucleotidyltransferase activity of NcRVT pre-incubated with α-^{32}P-dCTP in the presence of Mn^{2+}. The reaction was chased with dNTP (dN), NTP (rN), ATP (A), UTP (U), CTP (C), or GTP (G). (**E**) Activity of His-tagged wild-type *rvt* (G0022) and the D529A mutant (G0021) in five consecutive peak fractions from the sucrose gradient. NcRVT was pre-incubated with α-^{32}P-dCTP in the presence of Mn^{2+}, and the reactions were chased with CTP. The top panel compares the amount of wild-type and mutant protein by Western blotting of the same fractions probed with anti-His antibody.

Fig. 4. Relation of *rvt* genes to other RT classes. (**A**) A phylogram indicating both minimum evolution (ME) and maximum likelihood (ML) support values for the most basal branches, and ME support for each colored clade in cases where it exceeds 70%. (**B**) A NeighborNet phylogenetic network built using protein ML distances under WAG model with SplitsTree 4.10 (see Methods).


coiled-coil
1 2
3(A)
4(B)
5(C)
6(D)
7(E)
Thumb
RVT
2a
D
DD
GGLG
nLTR
AP
REL
TERT
TEN
TRBD
PLE
GIY-YIG
Group II
X
HNH
Retron
LTR/RV
PR
RH
IN
DIRS
RH
YR
vRdRP
PR [MT]


Herpetosiphon
env1
env2
Bacteria
Saprolegnia parasitica 1
Saprolegnia parasitica 2
Pythium ultimum
Phytophthora ramorum
Phytophthora sojae
Phytophthora capsici
Phytophthora infestans
Stramenopiles
Adineta vaga
Rotifer A
Onychiurus arcticus
Vavraia culicis
Microsporidia
Allomyces macrogynus 1
Allomyces macrogynus 2
Chytrids
Macrotrachela quadricornifera
Philodina roseola
Rotifer B
Fomitiporia mediterranea 1
Fomitiporia mediterranea 2
Fomitiporia mediterranea 3
Bas
Schizophyllum commune
Amanita bisporigera
Laccaria bicolor
Pleurotus ostreatus 1
Pleurotus ostreatus 2*
Bas
Physcomitrella patens
Plant
Tuber melanosporum 2
Tuber melanosporum 3*
Tuber melanosporum 1
Pez
Uncinocarpus reesii
Coccidioides immitis
Paracoccidioides brasiliensis
Ajellomyces capsulatus
Ajellomyces dermatitidis
Penicillum marneffei
Talaromyces stipitatus
Penicillum chrysogenum
Aspergillus nidulans
Aspergillus fumigatus
Neosartorya fischeri
Aspergillus oryzae
Aspergillus terreus
Aspergillus niger
Aspergillus carbonarius
Eur
Ts
Phaeosphaeria nodorum
Ustilago maydis
M. anisopliae
Phaeosphaeria nodorum
P.nod.
P. tri.
L.mac.
Lm
Uncinocarpus reesii
Coccidioides immitis
P. brasiliensis
Ajellomyces capsulatus
Ajellomyces dermatitidis
Eur
Gibberella moniliformis
Fusarium oxysporum
Chaetomium globosum
Thielavia terrestris
M. thermophila
Podospora anserina
Sor
Pa
Aspergillus nidulans
Aspergillus oryzae
Aspergillus terreus
Aspergillus carbonarius
Aspergillus niger
Eur
Nectria haematococca
Gibberella zeae
Fusarium oxysporum
Gibberella moniliformis
Cryphonectria parasitica
Magnaporthe oryzae
Verticillium dahliae
Verticillium albo-atrum
Sor
Mo
Phaeosphaeria nodorum*
Leptosphaeria maculans
Alternaria brassicicola
Cochliobolus heterostrophus
Pyrenophora tritici-repentis
Pyrenophora teres
Dot
Aspergillus oryzae
Aspergillus terreus
Aspergillus nidulans
Eur
Sclerotinia sclerotiorum
Botryotinia fuckeliana
Leo
Geomyces destructans
Botryotinia fuckeliana
Sclerotinia sclerotiorum
Leo
Glomerella graminicola
Nectria haematococca
Verticillium dahliae
Verticillium albo-atrum
Myceliophthora thermophila
Chaetomium globosum
Thielavia terrestris
Sordaria macrospora
Neurospora discreta
Neurospora crassa
Neurospora tetrasperma
Sor
Nc
0.2


A
His
3AT
RVT1
05498
06110
RVT1
RVT2
05498
pMau
BL
CHX
B
mm/hr
2225
2489
Δrvt1
BL
CHX
C
DEAE
D
-86
-46
-30
-25
-10
- dN rN A U C G
E
G0022 (wt)
G0021 (mut)


A
RTL
Retro-
plasmid
DGR
P
Retron
Group II
Intron
LINE-AP
Gypsy
L
Caulimovirus
Retrovirus
V
LINE-REL
DIRS
Hepadnavirus
Copia
BEL
RVT
TERT
T
PLE
B
0.1
LINE
L
RVT
V
RTL
Retro-
plasmid
DGR
Group II
Intron
P
TERT
Retron
T
PLE