SURVEY AND SUMMARY
Role of RNA helicases in HIV-1 replication

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ABSTRACT
Viruses are replication competent genomes which are relatively gene-poor. Even the largest viruses (i.e. Herpesviruses) encode only slightly >200 open reading frames (ORFs). However, because viruses replicate obligatorily inside cells, and considering that evolution may be driven by a principle of economy of scale, it is reasonable to surmise that many viruses have evolved the ability to co-opt cell-encoded proteins to provide needed surrogate functions. An in silico survey of viral sequence databases reveals that most positive-strand and double-stranded RNA viruses have ORFs for RNA helicases. On the other hand, the genomes of retroviruses are devoid of virally-encoded helicase. Here, we review in brief the notion that the human immunodeficiency virus (HIV-1) has adopted the ability to use one or more cellular RNA helicases for its replicative life cycle.

INTRODUCTION
Helicases are enzymes that separate in an energy-dependent manner stretches of duplexed DNA and/or RNA into single-stranded components. Currently, based on characteristic motifs and the sequence comparisons, three superfamilies (SF1 through 3) and two smaller families (F4, F5) of helicases have been identified (1). Superfamily 1 and 2 contain helicases which share seven or more recognized signature amino acid motifs while SF3 and F4 and F5 helicases are characterized generally by three conserved motifs (2); the F4 and F5 proteins are largely bacterial and bacteriophage proteins. Currently, it should be cautioned that many ‘helicases’ are not bona fide helicases, but may only function as RNA translocases, perhaps to fulfill functions in the remodeling of ribonucleoprotein complexes (RNP).

DEAD-box and the related DEAH, DExH and DExD (3) helicases are the most numerous members of SF2 and are ubiquitously present in eukaryotic genomes. These helicases share eight conserved motifs and are commonly referred to as the DExH/D family of helicases. Humans, Arabidopsis and Saccharomyces have ~38, 55 and 25 such entities, respectively (4). Differing from DNA helicases and DExH proteins, DEAD helicases are poor in unwinding long nucleic acid duplexes and are best suited for separating short RNA hybrids. DEAD-box proteins bind with high-affinity RNA–protein complexes while exhibiting little RNA sequence preference. This suggests that the specificity determinants for DEAD helicases may be through the recognition of protein factors. In this regard, a better understanding of the roles for DEAD proteins depends on the clear characterization of their respective interacting proteins.

Although the precise substrate for most helicases awaits definition, DEAD helicases are generally thought to participate pleiotropically in many aspects of RNA metabolism including transcription, mRNA splicing, mRNA export, translation, RNA stability and mitochondrial gene expression (5–8). Some examples of helicases and their attributed functions include the following. UAP56, Brr2, Prp16, Prp22 and Prp43 play roles in RNA-splicing (4,9), while Dbp5 (10,11) and DDX3 (12) chaperone RNAs from the nucleus into the cytoplasm. elf4a and Ded1 serve for translation of mRNAs while Rh1B, Ski2, Dob1, Dh1 helicases contribute to mRNA stability (4). Other DEAD helicases act in ribosome biogenesis through regulation of small nucleolar RNAs and ribosomal RNAs (rRNAs) interactions (13,14). Finally, Neurospora and Trypanosoma DEAD proteins contribute to mitochondrial gene expression (15,16); a Cryptooccocus DEAD helicase is required for cryptococcosis pathogenesis (17), and the dipteran Chironomus tentans uses a hrp84 DEAD helicase to regulate mRNA transport from the nucleus into the cytoplasm onto polyribosomes (18).

Given that helicases significantly contribute to normal cellular metabolism, are they similarly essential to viruses? The operational answer appears to be a qualified ‘yes’. Indeed, when DEAD/DEAH-box helicase motif (InterPro IPR001410) was used to search the EMBL-EBI database, 1561 matches to individual viral sequence entries were found (http://www.ebi.ac.uk/interpro/DisplayIproEntry?ac=IPR001410), suggesting that many viruses have evolved to encode directly helicase or helicase-like proteins. The strongest biological evidence which supports the importance of a helicase in the virus life cycle comes from those viruses with an RNA genome. Hence, all positive-strand RNA viruses encode one or more helicase/helicase-like open reading frame (ORF) which, aside from the RNA-dependant RNA polymerase, is the most highly conserved viral sequence.
Although less ubiquitous, helicases are also found in other types of viruses (see some examples listed in Table 1). Direct mutagenesis studies have confirmed that a helicase function is biologically required for the replication of many viruses including vaccinia virus (19), poliovirus (20), alphaviruses (21), brome mosaic virus (22), nidoviruses (23,24) and flaviviruses (25–27).

### ROLE OF HELICASE IN THE HIV-1 LIFE CYCLE

In 1981, the first cases of acquired immunodeficiency syndrome (AIDS) were described in American homosexual men. Thereafter, within three short years, French and American scientists confirmed that the human immunodeficiency virus (HIV) is the causative agent for AIDS. In the ensuing 20 years, >20 million individuals have died from AIDS; and currently, in 2006, ~50 million people worldwide are infected by HIV-1 with 3 million incremental AIDS deaths and 4–5 million new infections occurring annually. The magnitude of this burden casts urgency to medical research on HIV/AIDS.

HIV-1 is a retrovirus of the lentivirus genus with an RNA genome of ~9 kilobases which encodes nine polypeptides. The major HIV-1 structural proteins are encoded by three genes, *gag* (group-specific antigen), *pol* (polymerase) and *env* (envelope), while the accessory proteins, *Vif*, *Vpu*, *Vpr* and *Nef*, and the regulatory proteins, *Tat* and *Rev*, are the primary translation products of multiply-spliced mRNA. HIV-1 infects CD4+ human T-cells and macrophages and integrates as a provirus into the host cell’s DNA. Gene expression of HIV-1 is governed transcriptionally by a viral protein, *Tat* (28,29), via its binding to a nascent viral TAR RNA (30), and post- transcriptionally by a second viral protein *Rev* (31,32) through its association with the viral RRE RNA. Both *Tat* and *Rev* interact with several host cell proteins in their transcriptional and post-transcriptional functions (33). HIV-1 does not encode for any RNA helicase; however, findings suggest that host cell RNA helicases may be involved in the reverse transcription of HIV-1 RNA, in HIV-1 mRNA transcription and in the nucleus-to-cytoplasm transport of viral mRNA.

A recent unexpected finding revealed the possibility that an RNA helicase may potentially contribute roles in HIV-1 particle assembly and reverse transcription (34). Using proteomic analyses, Roy *et al.* (34) reported that the DEAH protein RNA helicase A (RHA) was found associated with HIV-1 Gag and packaged into HIV-1 virions in an RNA-dependent manner. When RHA was knocked down in cells, HIV-1 particles which were produced from these cells were significantly less infectious. This appears to be compatible with two possible explanations. First, it is conceivable that RHA participates in the formation of infectious virus particles either by shaping Gag–RNA interaction during viral particle assembly or by budding. Failure of RHA to properly restructure viral RNP could explain the observed reduced infectivity. Second, Roy *et al.* (34) reported evidence that HIV-1 particles that do not contain RHA showed reduced virion-endogenous reverse transcriptase activity. In this respect, it may be that RHA assists HIV-1 reverse transcriptase to more efficiently copy RNA by unwinding RNA secondary structure or by promoting the interaction of viral RNA with the nucleocapsid protein in order to assemble a better reverse transcription complex.

Separate from reverse transcription, the unwinding of highly structured RNAs might also be reasoned to be important for transcription (35). However, direct evidence for an RNA helicase role has been somewhat elusive. There are several examples which seemingly support an activity for RNA helicase in transcription. First, in vaccinia virus, it has been postulated that the NPH-II helicase assists transcription by strand-separating duplexed RNA structures to prevent R-loop formation behind the elongating RNA polymerase (36). Second, RHA has been invoked to provide a factor-recruitment role, bridging at the promoter the CREB-binding protein (37) and RNA polymerase II (37). Third, the p68 DEAD-box helicase was shown recently to be a novel transcriptional co-activator for p53’s transcriptional function (38). Interestingly, in the latter two instances, neither the ATPase nor the helicase activity of RHA and p68 is apparently required for their attributed transcriptional roles.

For HIV-1, two recent studies provide clues that RNA helicases may also serve co-factor function for transcription from the viral long terminal repeat (LTR). Fuji *et al.* (39) observed that RHA conserves in its N-terminus two double-stranded RNA-binding (dsRBD) domains characterized previously for the TAR RNA-binding protein, TRBP (40,41). These investigators found in both reporter and virus replication assays that RHA activated, in a TAR RNA-binding dependant manner, HIV-1 LTR-directed transcription (39). Next, Coco *et al.* (42) separately described that the expression of a DExH RNA helicase, RH116, was significantly induced after infection of HeLa-C4 cells by HIV-1. RH116 was found localized in the nucleus of HIV-1 infected cells and was observed to augment the transcription of unspliced HIV-1 transcripts. While both studies offer tantalizing evidence for roles by two different RNA helicases in HIV-1 transcription, critical details on how the helicases function by binding to TAR RNA and how this binding might cooperate with the activities of the viral transcriptional activator, Tat, are lacking. Pending more direct experimental tests, it remains unclear whether RHA and RH116 provide direct

| Sources | Superfamily 1 | Superfamily 2 | Superfamily 3 |
|---------|--------------|--------------|--------------|
| Animal DNA virus | U5 (Herpesvirus) | UL9 (Herpesvirus) | E1 (91) T antigen (SV40) |
| Animal RNA virus | NS2 (Alphavirus) p70 (Rubella virus) | NS3 (Flavivirus) Nsp3 (Hepatitis C virus) | NS3 (Parvovirus) |
| | NPH-II (Poxvirus) | (Hepatitis E) | (Rhinovirus) |
roles in transcription or indirectly influence the milieu of polymerase II initiation/elongation at the LTR.

Downstream from transcription, the fate of HIV-1 encoded RNA is regulated at the step of export of unspliced/partially spliced moieties from the nucleus into the cytoplasm. Unspliced and partially spliced viral RNAs code for genomic RNAs that are packaged into progeny virions and structural proteins. Hence, the egress of these RNAs from the nucleus into the cytoplasm is critical to the life cycle of the virus. Exit of HIV RNAs from the nucleus is a significant issue because unspliced/partially spliced cellular mRNAs are routinely retained in and not permitted export from the nucleus (43–46). A large body of work has suggested an elegant solution to this conundrum. Thus, it was established that the HIV-1 encoded Rev protein binds a highly secondary structured element (Rev responsive element; RRE) present in all unspliced and partially spliced HIV transcripts (47–57); and this binding specifically distinguishes, for purposes of nuclear export, viral transcripts from cellular RNAs.

New evidence now suggests that RNA helicases are also co-factors for Rev-directed export of HIV-1 mRNAs (58). In its role of transporting unspliced and incompletely spliced viral RNAs from the nucleus, Rev directly interacts with nuclear export receptor CRM1 (59,60), and CRM1 is required for Rev-mediated export of HIV RNAs (59,61,62). A recent report provides data that an RNA helicase, DDX3, is an additional player in the Rev–CRM1–RRE complex (12). Thus, it was shown that DDX3 over-expression enhanced Rev-dependent, but not other export, pathway; and that DDX3 is a nucleo-cytoplasmic shuttling protein which binds CRM1 and Rev. Moreover, DDX3’s necessity for Rev/RRE/CRM1 function was demonstrated by knock-down of cell endogenous DDX3. Finally, because DDX3 locates to nuclear pore complexes (NPC), Yedavalli et al. (12) further proposed that this human helicase, like the analogous yeast Dpb5p (11), may function with Rev/CRM1 to remodel and ‘thread’ large unspliced HIV-1 RNAs through the nuclear pore, facilitating their final release to the cytoplasmic side of the NPC.

The above DDX3 results are consistent with two additional papers which described similar findings for a related RNA helicase, DDX1. Thus, Pomerantz and co-workers (63) showed that DDX1 binds directly to the N-terminus of Rev and to the RRE-RNA motif and participates in the export of unspliced HIV-1 RNA from the nucleus to the cytoplasm. Additionally, they illustrated that reduced expression of DDX1 in astrocyes explains the previously observed tissue restricted function of HIV-1 Rev (64). Fully spliced viral mRNAs encoding for viral Tat, Rev and Nef, proteins have been shown previously to exit the nucleus using the cellular mRNA export pathway. Export of these mRNA may require the RNA helicase Dpb5 (65,66). As yet, the involvement of Dpb5 in export of spliced HIV-1 viral RNA has not been fully clarified.

The story of HIV-1 and RNA helicases is, however, likely to be more complex than and unlikely to conclude simply with RHA, RH116, DDX1 and DDX3 (Figure 1). HIV-1 RNAs are extensively regulated through splicing. Splicing is a multiple-step process requiring the recognition of splice sites by spliceosomes. It is generally believed that remodeling of RNA–RNA and RNA–protein interactions within the spliceosome is catalyzed by a family of DEAD/DExH box RNA helicases. To date, seven mammalian proteins that are RNA helicases have been implicated in mRNA splicing (67,68). Whether there is specific preference by subclasses of RNA helicases for viral mRNA splicing remains to be clarified. Moreover, how cellular RNA helicases might contribute to the translation of viral mRNAs also require further investigation.

Recently both Van’t Wout et al. (69) and Krishnan and Zeichner (70) have provided evidence that the expression of several cellular RNA helicases including DDX24, DDX21, DDX18, DDX11 and DDX9 is modulated during HIV-1 infection; however, the precise cellular role and significance of these helicases for HIV-1 pathogenesis have not be characterized. Interestingly, Krishnan and Zeichner reported microarray data which examined the transition of HIV-1 infection from latency to productive replication, and found that several cellular RNA helicases were upregulated (71). For future understanding of functions, it will be important to design experiments which can segregate helicases which serve direct, although perhaps overlapping and redundant, roles on HIV-1 from those that might participate indirectly in the viral life cycle. Nevertheless, the convergence of evidence would support that several discrete cellular RNA helicases contribute importantly to the efficient execution of several steps in the HIV-1 replicative cycle.

**HELICASE INHIBITORS AS FUTURE ANTIVIRALS?**

Given that the HIV-1/AIDS disease burden has reached pandemic global proportions, new antiviral strategies that target molecularly delineated mechanisms used by this virus are urgently needed (72). Is there a possibility that host cell helicases can be therapeutic targets for anti-HIV-1 chemotherapy? Implicit within this question is the concept that one could attack a host cell protein in order to treat an infecting pathogen. Although targeting a cellular protein involved in a viral pathway risks obvious cytotoxicity, this approach avoids the inherent problem posed by rapid HIV-1 mutation to all currently utilized chemotherapeutics targeted to virus-encoded proteins. We note that inhibition of cell-encoded enzymes in medical therapy is not an unprecedented strategy. Suppression of angiotensin-converting enzyme (ACE) is widely used to treat hypertension, congestive heart failure, myocardial infarction, endothelial dysfunction and renal disease (73). Elsewhere, aromatase inhibitors have been used to treat hormone-dependent breast cancer (74), and inhibitors of cellular secretory proteases are contemplated for Alzheimer’s disease (75). We recently inhibited the cellular polyprotein convertase, furin, at minimal toxicity to the cell in order to block HIV-1 replication (76). Thus, a priori exclusion of cellular helicase as an antiviral target is not warranted.

Guarded optimism that small molecule helicase inhibitors can be developed against viruses arises from encouraging progress in non-retroviral systems. Unlike HIV-1, human herpesviruses physically encode helicases. The herpes simplex virus UL5 and UL9 genes are helicases in superfamily 1 and 2, respectively (77). HSV UL5 together with UL8 and UL52 form a heterotrimeric helicase–primase complex.
responsible for unwinding duplex viral DNA at replication forks. Two recent studies provide proof-of-concept that the HSV helicase–primase can be targeted at low host cell toxicity by two new classes of drugs, amino-thiazolyphenylmolecules (78) and thiazole amide derivatives (79). In addition, other studies suggest that the NS3 protein, a RNA helicase encoded by Hepatitis C virus and related West Nile virus and Japanese Encephalitis virus can be targeted to inhibit viral replication (80–82). This conceptual break through in drug development is important because it indicates
that target discrimination between different helicases by small molecule inhibitors is possible. Of relevance to HIV-1, a synthetic immunomodulator Murabutide was shown recently to suppress HIV-1 replication in macrophages and T cells. Murabutide was shown to inhibit the activity of RNA helicase RH116, blocking its positive transcriptional activity for HIV-1 gene expression (42).

If one looks beyond the signature motifs conserved amongst helicases, then it becomes clear that the different proteins are widely divergent in their coding sequences. In principle, this suggests that individual helicases can be abrogated with specificity in a knowledge-directed manner.

In theory, a helicase can be attacked by (i) inhibition of NTPase activity through direct competition for NTP binding, (ii) inhibition of substrate binding through direct competition at active site, (iii) allosteric mechanisms to affect NTP-binding/NTP hydrolysis and/or polynucleotide binding, and (iv) inhibition of unwinding activity by steric hinderance of helicase translocation along the polynucleotide substrate (83,84). Because the NTP-binding and substrate-binding pockets may be sufficiently similar between various helicases, specificity of inhibition through these sites will likely be extremely difficult, although perhaps not impossible. On the other hand, the tremendous variations in sequence and

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**Figure 2.** Involvement of Dicer RNA helicase, TRBP and HIV-1 TAR RNA in the biology of the cell’s micro RNA pathway. (A) Domain structure of Dicer proteins from mammals and *Drosophila*. (B) A schematic representation of a potential mechanism for TAR RNA-dependent suppression of Dicer activity. TAR RNA is suggested to bind to TRBP and decoy it from Dicer preventing Dicer-dependent miRNA processing activity.
sizes of helicases, in their oligomerization states, in their
discrete domains responsible for protein–protein interactions
and/or for targeting to specific nucleic acids (85), and in
their differential localizations within cells (86) offer interven-
tional possibilities outside of the NTP- or polynucleotide-
 binding sites.

We are in the preliminary stages of screening ring-
expanded nucleoside analogs found previously to be success-
ful NTPase/helicase inhibitors of West Nile virus, Hepatitis C
virus and Japanese encephalitis virus (81,82). We have
observed that a few of these candidate inhibitors have
substantial anti-HIV-1 activity at doses that do not incur
cytotoxicity to cells treated in tissue culture for 1 week.
Further studies are needed before concluding that these
compounds exert specific inhibition of DDX3, one of the
other cellular helicases, or some other target altogether.

CONCLUDING REMARKS AND PERSPECTIVES

There is another area where a cellular helicase activity and
HIV-1 are likely to intersect. An emerging research focus is
the role of small interfering RNAs (87) and microRNAs
(miRNA) as innate cell defenses against viruses including
HIV-1 (87–90). In human cells, the precursor for miRNA
(pre-miRNA) is processed by DICER (Figure 2A) which is
a ribonuclease with a bona fide RNA helicase domain
(91,92). A surprising recent finding revealed that the human
TRBP, which has been shown to be a potent binder of the
HIV-1 TAR RNA RNA (40,41), is an indispensable
dsRNA-binding partner of DICER which allows the latter
to associate with pre-miRNA (91,92). Without TRBP,
DICER’s miRNA processing activity is lost. Thus an in-
triguing scenario can potentially unfold. Accordingly, whereas
the ribonuclease–helicase protein DICER requires TRBP to
process duplex-structured miRNAs in order that the cell can
use such matured miRNAs for antiviral defense, it could be
speculated that HIV-1 has evolved to restrict this defense
by the ability to transcribe viral TAR RNA to squelch
TRBP away from DICER (Y. Bennasser, M. L. Yeung and
K. T. Jeang, manuscript submitted) (Figure 2B). If this think-
ing is correct, then HIV-1 has developed mechanisms not
only to co-opt the active functions of a virus-propitious cellu-
lar helicase (i.e. DDX3) but also to inactivate the role of a
second virus-pernicious helicase (i.e. DICER) for purposes
of selfish gain.

In a separate perspective, virus infection can trigger
through double-stranded viral RNAs an innate antiviral
immune response. Thus viral dsRNAs can be recognized by
cellular proteins [pattern-recognition receptors (PRRs)]
which initiate antiviral responses by inducing the production
of a variety of cytokines including type I interferons (IFN-α
and IFN-β) and initiating additional inflammatory and
adaptive immune responses. Recently, DExD/H RNA helicases
such as RIG-1 (retinoic acid inducible gene-1) (93) and
Mda5 (melanoma differentiation-associated gene 5) (94)
have been identified as suppressors of viral replication by
binding to virus associated dsRNA and activating type I
interferon-dependent antiviral immunity. Over-expression of
RIG-1 and Mda5 was found to enhance dsRNA induced
type I interferon antiviral response. Currently, it remains
speculative whether helicases like RIG-1 and Mda5 may rec-
ognize HIV-1 dsRNA and trigger an innate immune response.
Intriguingly, several reports exist in the literature that HIV-1
infection does induce activation of type 1 interferons (95,96).

In conclusion, by studying helicase proteins one can gain
insights into normal cellular metabolic processes, abnormal
inherited human diseases (e.g. Bloom syndrome, Werner syn-
drome, Cockayne’s syndrome and xeroderma pigmentosum;
all diseases with mutations in cellular helicases), and remark-
ably also the biology of viruses.

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