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MINIREVIEW

Virus-Encoded RNA Helicases

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Modulation of RNA structure is an essential step in many fundamental processes, including RNA synthesis, splicing, replication, and translation. Recently, an increasing number of putative RNA helicases from different organisms ranging from bacteria to yeast, humans, and viruses have been identified. Based on computer-assisted sequence comparisons, these proteins were grouped into a loosely defined "helicase-like" family. Some of the better-characterized members have been shown to possess nucleotide binding and hydrolysis activities, the capacity to interact with nucleic acids, and for only a few members, a nucleoside triphosphate (NTP)-dependent RNA helicase activity. Helicases are capable of enzymatically unwinding duplex RNA structures by disrupting the hydrogen bonds that maintain the two strands together. This is accomplished in a reaction that is coupled with the hydrolysis of an NTP, and thus all helicases described to date also have NTPase activities. Presumably, during NTP hydrolysis the energy released is utilized in the unwinding reaction, although it is not yet known with certainty how the two reactions are coupled.

In view of the importance of modulating RNA structures in diverse metabolic processes, RNA helicases are probably ubiquitous. Furthermore, such enzymes are most likely of key importance in the life cycle of viruses whose genomes are composed of RNA. Paradoxically, the viral helicase-like enzymes are among the latest newcomers in the vast family of putative RNA helicases; the information about them is still limited. The aim of this review is to focus on virus-encoded RNA helicases.

THREE SUPERFAMILIES OF PUTATIVE RNA HELICASES

Computer-assisted searches for sequence similarities have identified common amino acid motifs in proteins either demonstrated or postulated to be capable of NTP binding and hydrolysis and nucleic acid unwinding activities (12, 23). Based on sequence comparisons, three superfamilies (SF) of helicases have been defined (Table 1); these include the NTP-binding motif-containing proteins encoded by most positive-stranded RNA viruses (12, 14, 15). Each of these three SF includes cellular and viral DNA and RNA helicases, but only the viral RNA helicases will be discussed here. In SF1 are classified the alphavirus-like (nsP2-like) proteins; SF2 includes polypeptides similar to those encoded by the potyvirus-flavivirus-pestivirus groups (NS3-like proteins) and the helicase of vaccinia virus; SF3 includes the picornavirus-like (2C-like) proteins (14, 33). Interestingly, although the genomic organization of potyviruses is similar to that of the other members of the picorna-like viruses, their helicase proteins are more closely related to the corresponding flavivirus- and pestivirus-encoded proteins. Simian virus 40 large tumor antigen (SV40 T antigen) is also considered to be related to SF3 proteins (15).

The functional predictions have been corroborated by biochemical studies of several viral proteins classified among the three SF. However, for certain other viruses, such studies so far failed to confirm the postulated function.

In the alphavirus-like supergroup (members of SF1), the nsP2 protein of Semliki Forest virus (SFV) has been reported to possess NTPase activity (49). Similar activities have recently been shown to be associated with the nonstructural polyprotein of turnip yellow mosaic tymovirus (26) and of rubella virus (18). However, RNA helicase activity has thus far not been demonstrated for any of the NTP-binding helicase motif-containing proteins of viruses belonging to SF1 (references 26 and 49 and our unpublished results). The possibility remains that these proteins exhibit helicase activity only when complexed with other viral and/or cellular proteins.

In the picornavirus-like supergroup (members of SF3), the poliovirus 2C protein exhibits NTPase activity (41, 50), but a helicase activity could not be demonstrated (50). Mutations of the NTP-binding motif in the cowpea mosaic virus (another member of SF3) B polyprotein rendered the virus unable to replicate in protoplasts (46). The SV40 T antigen is the only member of SF3 with a well-characterized DNA and RNA helicase activity (53, 57).

Among the members of SF2 (Table 1), considerable biochemical data are available to substantiate the motif-based predictions of enzymatic activities. RNA-stimulated NTPase activity has been demonstrated for the cylindrical inclusion (CI) protein of plum pox potyvirus (PPV) (32) and tamarillo mosaic potyvirus (TaMV) (5). It has also been shown for the NS3 protein of the flaviviruses West Nile virus (WNV) (64) and yellow fever virus (63), the NS3 protein of hepatitis C virus (HCV) (58), and the NS3 protein of bovine viral diarrhea pestivirus (BVDV) (59). A well-studied RNA-stimulated NTPase is the NTP phosphohydrolase II (NPH-II) of vaccinia virus (56). Moreover, an RNA helicase activity has been demonstrated for all the SF2 viruses mentioned above (5, 25, 27, 32, 62), except for WNV.

CONSERVED MOTIFS

The clearest common denominator of the three SF is the NTP-binding motif first characterized by Walker et al. (61). This motif is composed of the A and B sites (Fig. 1). The A site consists of a stretch of hydrophobic residues followed by the conserved sequence GxxxxGKS/T, where x is any amino acid. The B site consists of an Asp preceded by a stretch of hydrophobic amino acids (13). X-ray crystallography data have shown that the A site is directly involved in binding of the β and γ phosphates of the NTP, while the B site serves to chelate the Mg^{2+} of the Mg-NTP complex. Mutations in either the A

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TABLE 1. Proposed SF of virus-encoded NTP-helicase motif-containing proteins[a]

	SF1	Protein[b]	SF2	Protein[b]	SF3	Protein[b]
Positive-stranded plant RNA viruses	Alfamovirus	1a	Bymovirus	p270	Comovirus	p58
	Bromovirus	1a	Potyvirus	CI[c]	Nepovirus	p72
	Capillovirus	p241			Sequivirus	p336
	Carlavirus	p26			Waikavirus	ORF 1
	Closterovirus	p295				
	Cucumovirus	1a				
	Furovirus	p237				
	Hordeivirus	p130				
	Idaeovirus	p190				
	Potexvirus	p180				
	Tobamovirus	p126				
	Tobravirus	p134				
	Tymovirus	p206				
Positive-stranded animal RNA viruses	Alphavirus	nsP2	Flavivirus	NS3[c]	Aphtovirus	2C
	Arterivirus	p159	Hepatitis C virus	NS3[c]	Calicivirus	p257
	Coronavirus	ORF-1b	Pestivirus	NS3[c]	Cardiovirus	2C
	Hepatitis E virus	ORF-1			Enterovirus	2C
	Rubivirus	p220			Hepatovirus	2C
					Rhinovirus	2C
Double-stranded RNA viruses			HAV[d]	ORF B		
DNA plant viruses					Geminivirus	AL1
DNA animal viruses	Herpesvirus	BBLF4, gp55, UL5[c]	Herpesvirus	gp51, UL9[c]	Papovavirus	T antigen,[c] E1
			Poxvirus	NPH-I, II[c]	Parvovirus	NS1[c]

[a] The classification is an updated version of the previously published versions (14, 15, 30). The nomenclature of the virus groups was established by Fauquet and Martelli (6). For viruses encoding a second helicase-like domain in the TGB, only the helicase within the replicative protein is represented. Adenoviruses, reoviruses, and coconut foliar decay virus are not included since they do not contain a complete set of helicase motifs.

[b] The virus protein containing the NTP-helicase motif exemplified by the protein of the type-member virus whenever available.

[c] Established helicase activity.

[d] HAV, hypovirulence-associated virus of chestnut blight fungus.

or B site dramatically affected the ATPase and helicase activities of vaccinia virus NPH-II (19) and the best-characterized RNA helicase, the eukaryotic translation initiation factor eIF-4A (45). It should be recalled that the presence of the NTP-binding motif is shared by all helicases and also by a wide variety of other NTP-utilizing enzymes (12, 61), and thus additional motifs are necessary to compose the helicase signature.

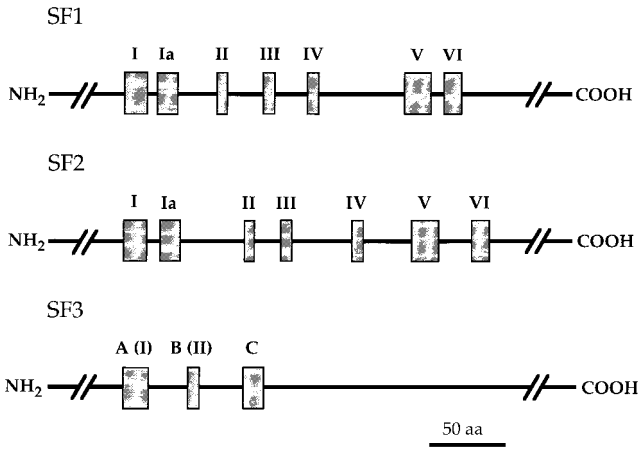

FIG. 1. Diagram representing the conserved motifs of the three SF of helicases. The sizes of the conserved motifs and the distances between them are drawn to scale based on the average number of aligned amino acids (aa) (30). Motifs I (A) and II (B) together comprise the purine NTP-binding motif.

Altogether SF1 and SF2 share seven conserved segments (Fig. 1; Table 2) designated I (or A site), Ia, and II (or B site) through VI, arranged in a colinear fashion. Segments Ia, III, and IV are the least conserved; no potential function has yet been assigned to them. Segment VI, together with segments I, II, and V, is the best conserved. Based on the abundance of basic residues, notably Arg, motif VI has been proposed to bind nucleic acids. In the case of PPV, Fernández et al. (7) have shown that this region is indeed necessary and sufficient to confer RNA-binding capacity to the CI protein. Mutations in the corresponding region of eIF-4A abolished binding to RNA and reduced the ATPase activity to low levels. The helicase activity of these mutants was severely impaired, suggesting coupling of ATP hydrolysis and RNA unwinding (44). Individual mutations in motif VI in the NPH-II of vaccinia virus caused severe defects in ATP hydrolysis and RNA unwinding but did not affect RNA binding (19). Thus, it seems the contribution of the conserved helicase motifs to overall protein function is context dependent. These results are consistent with the implication of motif VI in the ATPase activity rather than only in interaction with nucleic acids.

SF3 proteins contain only three conserved segments (15), including in each case the A and B sites and a third consensus sequence designated C (Fig. 1).

HELICASE ASSAY

The accepted assay for helicase activity includes an RNA substrate that is susceptible to unwinding, consisting of two annealed complementary RNA strands, of which one is radio-

labeled. This substrate is incubated with a (putative) helicase in the presence of an NTP and a divalent cation (usually Mg^{2+}), and the products of the unwinding reaction are resolved on a sodium dodecyl sulfate-polyacrylamide gel. Since single-stranded RNA migrates more rapidly in the gel than does the unwound duplex, the displaced strand is directly observed. The longer of the two strands is usually referred to as the template strand, the shorter strand is referred to as the released (or displaced) strand. One of the strands (usually the template strand) may be circular. The substrate can be altered in different ways: the length can be changed, nonhomologous tails can be added, or a second strand to be displaced can be added to the first one. In the latter situation, the strands can be composed of RNA, DNA, or of a DNA/RNA hybrid.

CHARACTERISTICS OF VIRAL RNA HELICASES

Nucleic acid-stimulated NTPase activity. All established viral helicases exhibit NTPase activity. This activity is dependent on the presence of an NTP and of a divalent cation, commonly Mg^{2+}. The products of NTP hydrolysis for all helicases studied are NDP and P*i*, and hydrolysis is a sine qua non condition for the unwinding activity, since substitution of ATP by diverse nonhydrolyzable ATP analogs cannot support strand displacement (32, 56, 62).

In general the NTPase activity of helicases is stimulated by the presence of single-stranded nucleic acids. This may be expected of an enzyme that remains bound to one RNA strand by the energy generated by ATP hydrolysis to separate hydrogen-bonded base pairs of a duplex structure. Furthermore, the NTPase activity of each kind of helicase (i.e., DNA or RNA helicase) is preferentially stimulated by the type of nucleic acid to which it is functionally related.

The bound nucleic acid could induce a protein conformation characterized by an improved fit of the active center of the NTPase domain for the NTP, leading to more efficient binding and hydrolysis of the nucleotide. Interestingly, NTPase activity is apparently not stimulated at high (100 to 200 mM) salt concentrations (5, 31, 32, 58, 59, 63). This may be due to the fact that in conditions of high ionic strength the nucleic acid cannot remain bound to the enzyme; alternatively, the nucleic acid remains bound to the enzyme, but the enzyme adopts a conformation that is no longer suitable for unwinding, as suggested for the helicase of SV40, the T antigen (53).

The level of nucleic acid stimulation of the ATPase activity varies among the members of the three SF of viral helicases. The highest stimulatory effect (3- to 15-fold) has been described for the enzymes belonging to SF2 (5, 32, 58, 59, 63, 64) whose helicase activity was firmly established. In contrast, for the ATPases of SFV, rubella virus, turnip yellow mosaic virus (members of SF1), and poliovirus (member of SF3), only a twofold stimulation was observed (18, 26, 49, 50) and no helicase activity has been demonstrated for these enzymes thus far. Could this difference in stimulatory effect merely reflect an SF feature or could it be related to the absence of a helicase activity?

Polarity. The polarity of unwinding can be determined by using an RNA substrate consisting of a linear single-stranded template RNA with duplex regions near both ends. As opposed to DNA helicases which function either in a 5′ to 3′ or a 3′ to 5′ direction (reviewed in reference 38), the viral RNA helicases described to date (5, 32, 53, 56, 62) drive unidirectional 3′ to 5′ unwinding of the RNA duplex region. (By convention the polarity of an unwinding reaction is defined with respect to the strand of nucleic acid to which the helicase is bound.) However, the possibility remains that other putative

TABLE 2. Conserved motifs of the three SF of viral helicases[a]

SF	Motif									
	I	Ia	II	III	IV	V	VI	A(I)	B(II)	C
SF1	bxGxPGxGKS/Tx_3b	Rxbu$_2$xPTRxux$_2$Eb	b$_3$DEb b$_4$DExH	b$_4$GDx$_2$Q bxuTATPP	ub$_2$uPS	Tbx$_3$QGxTbx$_2$Vxb$_2$ bubxTDbxExGuxbx$_4$u$_2$	bVAuTR Tx$_5$QRxGRuGR	EPbxb$_3$xGx$_2$GxGKS	Qxb$_2$ubDD	KGx$_2$*xSxbubxSTN
SF2	b$_2$x$_4$GSGKS/Tx_3bP									
SF3										

[a] The consensus motifs of the three helicase SF are derived from sequence alignments (30). In SF1, motifs Ia and IV are not shown since the consensus pattern is rather weak. The single letter code is used for the conserved amino acids. Abbreviations: b, bulky hydrophobic residues (aliphatic or aromatic); u, bulky aliphatic residues; *, aromatic residues; x, any residue.

RNA helicases could display a 5′ to 3′ direction of unwinding. Nevertheless, unidirectionality could constitute an intrinsic characteristic of RNA helicases of cellular as well as of viral origin. An interesting exception is eIF-4A, which operates in both directions (52). However, given that unwinding in both directions triggered by eIF-4A absolutely requires the presence of eIF-4B, bidirectionality does not seem to be an intrinsic property of eIF-4A or in general of RNA helicases.

Substrate requirements. The nature of the substrate appears important for the unwinding reaction. Binding of the RNA helicase to a region of unpaired nucleic acid is probably a prerequisite to initiate unwinding (8). Completely duplex regions are not unwound. Viral RNA helicases require a 3′ single-stranded region to which they first bind and then, either by a processive translocation or a cooperative binding mechanism, progress toward the duplex region of the substrate to accomplish strand displacement. In the case of the BVDV NS3 helicase (62) it has been elegantly shown that this anchoring role may be played either by a free 3′ terminus on the template strand or by an internal unpaired segment on the template RNA located closer to the 5′ end of the template strand.

Template specificity. To date, there have been no reports of specific RNA sequence requirements for viral RNA helicase activity in vitro. The viral RNA helicases characterized so far hydrolyze ATP in the presence of a variety of RNAs, including synthetic homopolymers, and, curiously, also in the presence of single-stranded DNA. Moreover, all these enzymes can bind single-stranded RNA and single-stranded DNA, albeit with different affinities. This low specificity distinguishes them from cellular RNA helicases, such as human p68 (22) or eIF-4A (17), whose ATPase activity is not stimulated by poly(dA) and for which no binding of single-stranded DNA is observed. However, even though the viral enzymes can be promiscuously stimulated and can bind to a range of nucleic acids, they cannot unwind DNA duplexes. Particular exceptions are provided by the SV40 T antigen and the vaccinia virus NPH-II that may act as DNA or RNA helicases depending on the context (1, 53–56).

Monomeric or oligomeric nature of helicases. Most mechanisms proposed for the helicase action anticipate that a functional helicase requires two features: an ATP-driven conformational rearrangement of the protein that can generate energy and a minimum of two nucleic acid-binding sites between which this energy can be exerted to promote unwinding (reviewed in reference 35). Although multiple binding sites might exist within a single polypeptide, oligomerization probably provides a simple mechanism by which helicases acquire multiple nucleic acid-binding sites. This requirement seems indispensable for the unwinding reaction, since it enables a helicase to bind either single-stranded and duplex regions of a polynucleotide simultaneously or two complementary single strands of an unwinding fork. Indeed, the majority of DNA helicases for which the state of protein assembly has been examined appear to form oligomeric structures (36). Such a propensity toward a multimeric state may be a general characteristic of RNA helicases as well. A striking illustration is eIF-4A, which functions as an RNA helicase only when dimerized with eIF-4B. On its own, eIF-4A possesses a single RNA-binding domain and there is ample evidence that eIF-4B serves to stabilize the eIF-4A/mRNA complex by donating its two RNA-binding domains (39, 40, 44, 52). Thus, the helicase must be supplied with additional RNA-binding domains either by accessory proteins, as in the case of eIF-4B, or by assembling identical subunits, as in the case of the SV40 T antigen. The SV40 T antigen helicase forms a double hexamer when bound to the origin of replication in the presence of ATP, and it forms a tetramer in the absence of ATP (37).

The CI RNA helicase of PPV has only one RNA-binding domain situated in the C-terminal part of the protein (7). In agreement with the model presented above, this protein purifies as a large complex (32), possibly as a multimer. On the other hand, two human nuclear RNA helicases, p68 and RNA helicase A, and the vaccinia virus NPH-II RNA helicase were reported to be active in a monomeric form in vitro (22, 34, 56). Since no experimental evidence is yet available concerning the number of RNA-binding domains of these three helicases, two hypotheses can be presented. The aforementioned proteins could possess more than one RNA-binding domain, implying that they are self-sufficient in acting as helicases in a monomeric form. Computer searches for profiles of RNA-binding domains have indeed highlighted two copies of such domains in each of the three helicases (9). Alternatively, enzymes that are monomeric in solution under some conditions could assemble to active oligomeric forms upon binding RNA or a nucleotide cofactor. Studies of DNA helicases strongly suggest that DNA and/or ATP are allosteric effectors of DNA helicases (35).

ESTABLISHED RNA HELICASE ACTIVITY OF DNA VIRUSES

Vaccinia virus NPH-II RNA helicase. Vaccinia virus, a large double-stranded DNA virus, encodes an essential RNA helicase with an as-yet-uncertain physiological role. The enzyme was purified from virions (56) and from cultured mammalian cells coinfected with two recombinant vaccinia viruses: one carrying a His-tagged copy of the NPH-II under the control of T7 promoter and the second expressing the T7 RNA polymerase (19). The biochemical properties of the recombinant protein were identical to those of the RNA helicase purified from virions. NTP hydrolysis was indispensable for strand displacement and neither adenosine 5′-[β,γ-methylene]triphosphate nor adenosine 5′-[β,γ-imido]triphosphate could substitute for ATP (56). The helicase activity also depended on the presence of a divalent cation: Mg^{2+}, Co^{2+}, and Mn^{2+} were effective cofactors (51). The enzyme displayed a low basal level (6 to 10%) of NTP hydrolysis in the absence of either single-stranded DNA or RNA. Recently, it was reported that NPH-II can also unwind DNA/DNA substrates, although less efficiently than RNA/RNA substrates (1); this however remains controversial (21), and differences in the structure of the DNA/DNA substrates used could account for this discrepancy (1). The polarity of RNA or DNA unwinding was exclusively 3′ to 5′.

The contributions of the different conserved motifs to enzyme activity were assessed by targeted mutagenesis of the recombinant (19) or native protein (20). The Lys-to-Ala mutant (replacement in the A site) and Asp-to-Ala or Glu-to-Ala mutants (in the B site) bound RNA as well as the wild-type protein but were severely defective in NTPase and helicase functions. Interestingly, the His-to-Ala mutant (in the B site) was active in RNA binding and NTP hydrolysis but was defective in duplex unwinding, suggesting that His in the B site is required to couple the NTPase and helicase activities. Individual point mutations in the conserved residues of motif VI had a significant negative impact on the NTPase activity per se that was correlated with defective helicase activity, but apparently RNA binding remained unaffected (20).

SV40 RNA helicase. The SV40 T antigen is a multifunctional protein with well-studied NTPase, nucleic acid binding, and both DNA and RNA helicase activities. It is required for rep-

lication of the SV40 genome and for regulation of viral and cellular genes (60).

The DNA helicase activity of the T antigen has been well characterized and convincingly related to its role in viral DNA replication. However this activity lies beyond the scope of the present review and will not be described here. The significance of its RNA helicase activity is as yet unclear; nevertheless, certain characteristics of this activity are reviewed here.

The large T antigen was purified from HeLa cells infected with a hybrid adeno-SV40 virus (57), and the properties of its DNA and RNA helicase activities were studied in vitro. Surprisingly, a major difference was noted while characterizing these two nucleic acid unwinding activities: even though the T antigen preferentially required ATP (or dATP) as the energy source for DNA unwinding, its RNA helicase was virtually nonfunctional in the presence of ATP. Conversely, either UTP, CTP, or GTP (as well as their deoxyribonucleotide counterparts) served as an efficient energy source in supporting RNA unwinding (53). Interestingly, using three different types of substrates, DNA/DNA, DNA/RNA, and RNA/RNA, it was shown that the nature of the single-stranded nucleic acid to which the T antigen helicase bound during initial complex formation determined whether ATP or non-ATP nucleotides could be used to fuel the unwinding reaction (53, 54). Substrates with overhanging 3′ single-stranded DNA required ATP as the energy source, regardless of whether the duplex region consisted of a DNA/DNA or DNA/RNA hybrid. Similarly, double-stranded regions in substrates with overhanging 3′ RNA tails were unwound in the presence of a non-ATP nucleotide. When tested in the absence of DNA or RNA, T antigen possessed a low basal level of ATPase or UTPase activity. However, single-stranded DNA stimulated hydrolysis of ATP severalfold, whereas single-stranded RNA strongly stimulated hydrolysis of non-ATP nucleotides (53).

As an interpretation of these results, Scheffner et al. (54) proposed a model which includes the following features. (i) When single-stranded DNA is bound to the T antigen, it could induce a protein conformation that is characterized by an improved fit of ATP relative to the active center of the helicase and bound ATP would be efficiently hydrolyzed. (ii) The conformational change in the protein caused by bound RNA could be subtly different, leading to improved binding and more efficient hydrolysis of UTP (or CTP or GTP). (iii) Finally, the finding that the anti-T monoclonal antibody Pab 204 inhibits both the DNA and RNA helicase reactions may indicate that the active center could be the same or overlap for both activities.

The RNA helicase activity required a 3′ single-stranded RNA extension to initiate unwinding in a 3′ to 5′ direction, just as in DNA unwinding (65). The T antigen seemed to be more efficient as a DNA helicase in terms of maximal substrate length capacity: although able to unwind duplex DNA of many thousand base pairs, it could unwind only short regions (<60 bp) of duplex RNA (53).

ESTABLISHED RNA HELICASE ACTIVITY OF RNA VIRUSES

All positive-stranded RNA virus-encoded helicases studied to date, i.e., PPV CI (32), TaMV CI (5), HCV NS3 (58), and BVDV NS3 (reference 62 and references therein), possess high levels of NTPase activity in the absence of nucleic acid; in this respect they differ from most cellular or DNA virus RNA helicases. Indeed, eIF-4A has almost no detectable ATPase activity in the absence of RNA (45); the same is true of human p68 (22), SV40 T antigen, and vaccinia virus NPH-II helicase. High basal NTPase activity could be an intrinsic characteristic of the helicases of positive-stranded RNA viruses.

PPV RNA helicase. The CI protein of potyviruses is the first positive-stranded RNA virus-encoded protein for which experimental evidence of RNA helicase activity was reported (32). The protein was purified from infected tobacco leaves (31, 32) and later expressed and purified as a fusion protein from bacteria (7). The native enzyme and the recombinant form exhibited indistinguishable biochemical properties.

The NTPase activity of the CI protein was stimulated by several types of nucleic acids with a preference for single-stranded RNA (31, 32). The helicase activity was absolutely dependent on the presence of NTP and Mg^{2+}. NTP hydrolysis was indispensable for strand displacement since adenosine 5′-[β,γ-methylene]triphosphate or adenosine 5′-[β,γ-imido]-triphosphate failed to support unwinding. The enzymatically separated RNA strands could be reannealed to reconstitute the initial substrate, indicating that RNA was not chemically modified during unwinding.

The CI protein was shown to efficiently bind poly(A) as well as poly(dA), even though the former was bound with higher affinity. In spite of this indiscriminate binding, the enzyme failed to unwind a partial DNA duplex in any of the conditions tested, suggesting that the helicase activity is specific for RNA substrates.

Substrate requirements of the CI protein were investigated using three types of RNA substrates: with a 3′, a 5′, or 3′ and 5′ overhangs. Only in the case of the substrates with a 5′ overhang was unwinding abolished. Thus, as for other RNA helicases the polarity of unwinding by the PPV helicase is 3′ to 5′.

The CI protein of another potyvirus, TaMV, purified from infected tobacco leaves (5), was shown to be an RNA helicase and to possess essentially the same characteristics as the PPV helicase.

BVDV NS3 helicase. The NS3 protein of BVDV was purified from baculovirus-infected insect cells (59). The recombinant protein possessed RNA-stimulated NTPase activity (59) and also RNA helicase activity (62). The RNA unwinding activity showed a preference for ATP and Mn^{2+}. Substitution of ATP by ATPγS eliminated strand displacement.

Although the NS3 protein bound single-stranded RNA and single-stranded DNA and was able to displace a DNA oligonucleotide from an RNA template, it could not unwind substrates in which the template strand consisted of DNA (62). This substrate specificity may indicate that the enzyme binds RNA with greater stability or that the conformation adopted when the RNA is bound is more favorable to ATP binding and hydrolysis.

The substrate requirements of the enzyme were evaluated using three different RNA substrates (62), duplex substrates possessing 3′ tails, 5′ tails, or no tail. The enzyme did not act on the two latter substrates, suggesting that the polarity of unwinding was 3′ to 5′ with respect to the template strand.

HCV NS3 helicase. The C-terminal region of the NS3 protein containing the NTPase-helicase domain was purified as a recombinant protein from *Escherichia coli*; it exhibited RNA-stimulated NTPase activity (25, 27, 58) and RNA helicase activity in the presence of ATP and of a divalent cation, either Mn^{2+} or Mg^{2+} (25, 27). The characteristics of this RNA helicase remain to be investigated.

DO VIRUSES NEED A HELICASE?

Helicases are a well-established class of enzymes that unwind double-stranded DNA, DNA/RNA hydrids, or double-stranded RNA structures during replication/transcription of

cellular and viral genomes. This activity might be involved in the replication of every virus, irrespective of the nature of its genome, since (partly) double-stranded polynucleotides have been shown to be present in the replicative cycle of all studied viruses. However, sequence analyses revealed a nonrandom distribution of (putative) helicases by different groups of viruses (12).

For viruses whose genome is composed of double-stranded DNA or RNA, the need of a helicase activity to disrupt the otherwise stable duplex template during replication and/or transcription elongation is easily conceivable. Indeed, the presence of a (putative) helicase is typical of all double-stranded DNA viruses for which the complete nucleotide sequence has been reported (12, 13). Although the sampling of sequenced double-stranded RNA viruses is as yet too small to draw relevant conclusions, viruses of this group with large genomes appear to contain helicase motifs. This is the case for the reoviruses (4, 43) and for the hypovirulence-associated virus of chestnut blight fungus (29, 55), as opposed to that for birnaviruses (12) whose small genomes contain no helicase motifs. Thus, a curious correlation seems to exist between genome size and the presence of putative helicases (12, 30).

The situation is somewhat different in the case of single-stranded viruses, in the sense that their genomes can be partially in duplex form. Moreover, during the replication of positive-stranded RNA viruses, the replication machinery must function in such a way that the templates are repeatedly available for a new round of replication. Thus, one hypothesis is that the replication machinery might require an RNA unwinding activity both to resolve intramolecular base pairing in the template RNA and to prevent the formation of extensive base pairing between template RNA and the nascent complementary strand.

About 80% of all positive-stranded RNA viruses whose genome has been sequenced encode at least one potential helicase. Only viruses with a relatively small genome (<5.8 kb) do not appear to encode a helicase (14, 28). It could be that RNA unwinding is not needed for replication of viruses with a small genome. There is an interesting exception to this observation: the human astrovirus (24) whose genome is ≈7.2 kb does not seem to encode a helicase.

Due to the paucity of current information concerning viral helicases, any attempt to assign a definite function to these enzymes in the life cycle of the virus remains, unfortunately, purely speculative. In spite of this hindrance three additional general hypotheses can be proposed. First, helicases might be responsible for the fidelity of replication, by involving a proofreading energy-dependent mechanism and thus reducing the error rate of the polymerase (28). This hypothesis might help to rationalize why viruses with relatively large genomes need a helicase to replicate efficiently. According to the second hypothesis, a helicase activity might be required for initiation of (early) transcription to strand separate double-stranded structures and/or during transcription elongation to prevent R-loop formation behind the elongating RNA polymerase, as has been suggested for the vaccinia virus helicase (21). A third hypothetical role for a virus-encoded helicase might be during initiation of translation. This model is inspired by the behavior of eIF-4A. In combination with eIF-4B, eIF-4A is believed to disrupt secondary structures in mRNA upstream of the initiation codon, thereby facilitating attachment of the 40S ribosome (52).

CONCLUSIONS

RNA helicases constitute an expanding group of proteins, as the signature motifs are observed in newly cloned genes from a wide spectrum of organisms. Based solely on sequence similarities and in the absence of supporting biochemical data, such gene products are typically designated putative helicases. Many of them probably have a helicase activity that depends on ATP hydrolysis and perform these reactions by very similar mechanisms. In the cases for which no helicase activity has been detected, one cannot rule out the possibility that the assay conditions employed to measure activity in vitro were inadequate. Yet in other cases, the enzyme may not be a helicase but may couple the energy generated from NTP hydrolysis to other processes, such as RNA encapsidation (41), cell-to-cell movement of the virus (51), or vesicular trafficking of replication complexes (50).

A group of positive-stranded RNA viruses of plants comprising the potex-, carla-, hordei-, and furoviruses, that contain a homologous triad of genes designated triple gene block (TGB) (42) constitute a remarkable example. The genome of these viruses possesses two copies of the helicase domain, one embedded in the replicative protein, presumably involved in RNA replication, and one mapping to the first protein of the TGB. This second helicase-like protein is required for movement of the virus in the infected plant but not for viral replication (2, 10, 47). Since viral movement is an active process, it is conceivable that it may involve an energy-dependent step. Indeed, for the TGB helicase-like protein of foxtail mosaic potexvirus, ATPase- and RNA-binding activities have been demonstrated (51). Both activities may be necessary to tag and/or sequester viral RNA molecules for their subsequent transport to neighboring cells.

The great diversity of functions achieved by variations of a common structural theme could be explained by the fact that helicase-like proteins differ in size and that in some of them additional N- and C-terminal sequences may determine the specificity of action of each protein. Such additional sequences would include membrane-targeting signals, as in the case of the poliovirus 2C protein (50) or the nuclear localization signals of the SFV nsP2 protein (48), or regions required to interact with accessory proteins with modulating roles.

From an evolutionary point of view, it has been proposed that the helicases of all three SF might comprise a relatively compact domain within the large class of proteins containing the NTP-binding motif and that the main branching point within this domain could have occurred before the eubacterial/eukaryotic divergence. This hypothesis is based on the fact that signatures of the three SF are encountered in prokaryotic and eukaryotic helicases, indicating that their common ancestor probably existed at a very early stage of evolution (11, 30).

Helicases have been extensively studied, especially DNA helicases that were discovered some 20 years ago. However, a number of important aspects of these enzymes remain unresolved. For example, the molecular mechanism of unwinding is still unknown for any helicase. In general, the proposed mechanism can be either passive or active (reviewed in references 35 and 36). In active models the protein plays a direct role in breaking the hydrogen bonds between base pairs, whereas in passive models the protein merely stabilizes the unwound state by binding to single-stranded regions of the polynucleotide. Another puzzling problem is that of energy transduction, i.e., the coupling of ATP binding and hydrolysis to polynucleotide unwinding. Identification of the intermediate helicase-nucleic acid complex would help to decipher the allosteric nature of this class of enzymes, the modulation of the relative affinities for DNA versus RNA as can be observed in the case of the versatile helicases of SV40 and of vaccinia virus, the interactions with their respective ligands, and how all these interactions are almost certainly modulated by the assembly state of

the helicase itself. In addition, further understanding of these and other aspects would require high-resolution structural information. To date no crystallographic data are available for any helicase. Hence, such studies would be useful in understanding the contribution of each conserved motif in the multiple biochemical activities displayed by a helicase or the role of additional domains flanking the core helicase domain.

Neither the retroviruses, the negative-stranded RNA viruses, nor certain single-stranded DNA viruses appear to encode a putative helicase. Because the reverse transcriptase-associated RNase H activity degrades the RNA strand of RNA/DNA hydrids (16) (reviewed in reference 3) and because a cellular helicase is probably responsible for the unwinding of RNA produced from the integrated proviral DNA, retroviruses would not require a helicase for their replication. As for the others, the tantalizing question remains as to whether they indeed do not require helicase action or whether they recruit cellular helicases for unwinding. The hypothesis that they may encode a noncanonical and still unidentified helicase cannot be excluded. Alternatively, these viruses might have resolved the problem of replication by a completely original and yet unknown mechanism without the assistance of helicases.

It will be important to identify the RNA species that in vivo are substrates for the virus-encoded RNA helicases. Among the helicases of this group which to date have been studied biochemically, none appears to require specific sequences. Presumably, such specificity exists in vivo and may result from a combination of sequence and secondary/tertiary structure of the RNA substrate. In this respect, these proteins and their corresponding substrates offer an exciting potential for drug development and for designing strategies for interfering with the viral infection cycle, since many of these proteins have already been shown to be important for the viability of the infectious agents.

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ADDENDUM IN PROOF

The crystal structure of the *Bacillus stearothermophilus* DNA helicase has recently been reported (H. S. Subramanya, L. E. Bird, J. A. Brannigan, and D. B. Wigley, Nature **384:**379–383, 1996).

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