

MINIREVIEW

Infectious Transcripts and cDNA Clones of RNA Viruses

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Received June 23, 1993; accepted November 3, 1993

Recombinant DNA technology makes it possible to analyze and modify genomes at the molecular level and thus gain deeper insight into their organization and expression. In this respect viruses, because of the small size of their genome, are particularly amenable to such investigations. In spite of this, the study of the molecular biology of nonretroviral RNA viruses has long been hampered by the fact that these viruses do not encompass a DNA intermediate step in their replication cycle. Furthermore, since to date the extremely varied and powerful molecular biology techniques aimed at modifying nucleic acids have been directed essentially at DNA substrates, new molecular tools had to be developed.

The possibility of obtaining infectious clones (as cDNAs or as *in vitro*-transcribed RNA copies) corresponding to the genomes of RNA viruses has greatly enhanced the potential of investigations. Indeed, they can facilitate studies of viruses that are present only in low titers in infected cells or whose isolation is problematic. Furthermore, and despite the relatively recent development of these tools, they have already provided precious information in the study of the genetic expression and replication of RNA viruses by the use of mutagenesis, deletions, insertions and by complementation experiments, but also in the study of natural or induced RNA recombination, of mechanisms generating defective-interfering RNAs or satellite RNAs, and of plant-virus interactions (such as the mechanisms of cell-to-cell movement). Moreover, these clones can also be considered as "pools" for viral genes or sequences of interest for the design of antiviral strategies and transcomplementation studies or for the development of new viral vectors. The molecular biology of

spherical plant RNA viruses has recently largely benefited from the availability of *in vitro*-produced infectious transcripts (reviewed in Bujarski and Miller, 1992).

We describe the different strategies employed to obtain infectious clones from nonretroviral RNA viruses (excluding viroids, satellites, and defective interfering RNAs) and discuss the different possible parameters affecting infectivity of such clones, keeping in mind, however, that the success or failure of the construction of these clones is often empirical and that very few systematic studies have been performed on the different parameters involved.

CONSTRUCTION OF INFECTIOUS CLONES

Full-length cDNA clones: Limitations and pitfalls

The construction of a full-length cDNA clone from which an infectious transcript can be synthesized *in vitro* is sometimes long and tedious. Although the presence of the entire viral sequence is generally thought to be required to obtain infectious clones, the possibility of producing infectious transcripts from incomplete viral cDNA clones has been reported (e.g., Davis *et al.*, 1989; Klump *et al.*, 1990) and warrants a word of caution. Moreover, cDNA synthesis, cloning strategies, and the design of sequences bordering the viral insert can have a strong influence on the infectivity of the resulting transcript.

It is generally admitted that the presence of nonviral nucleotides at the 5' end of viral transcripts strongly reduces infectivity. Furthermore, none of the commercially available *in vitro* transcription vectors leads to the production of transcripts devoid of vector-derived sequences.

A number of methods have been reported to produce long (>2 kb) cDNA fragments (Okayama and Berg, 1982; Gubler and Hoffman, 1983; Frankel and Friedmann, 1987; Schmid *et al.*, 1987), but none of them makes it possible to obtain full-length cDNAs without or with only very few nonviral nucleotides.

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Since in most cases the nucleotide sequence of the viral genomes considered has been determined, methods based on the use of viral-specific primers for DNA synthesis have been favored compared to those based on vector-primed cDNA synthesis, although an elegant combination of the two strategies has also been developed (Petty *et al.*, 1988).

In general, the construction of a cDNA consists of reverse transcribing the viral RNA into a single-stranded DNA using a primer hybridizing specifically to the 3' end of the viral genome. After elimination of the viral RNA, the single-stranded DNA is converted into the double-stranded form by initiating DNA synthesis with a second primer encompassing the sequence corresponding to the nucleotides at the 5' end of the viral RNA. Typically, the sequence for an RNA polymerase promoter fused to the viral sequence is included in the second primer.

A serious limiting factor for some viral genomes appears to be synthesis of the full-length first cDNA strand, probably because the polymerization step is hampered by strong secondary structures on the viral template RNA. It is noteworthy that even for a given virus, some research groups but not others have succeeded in obtaining a full-length first cDNA strand. This is the case for tobacco mosaic virus (TMV³; Meshi *et al.*, 1986; Dawson *et al.*, 1986; Holt and Beachy, 1991) and turnip yellow mosaic virus (TYMV; Weiland and Dreher, 1989; Skotnicki *et al.*, 1992; Boyer *et al.*, 1993). This discrepancy could result from the use of different strains or could reflect the importance of the sources of reagents employed.

Several variations on the general scheme for the production of full-length cDNA clones have been reported

and as a consequence, it is difficult to sort out an optimum protocol suitable for any virus. These variations are for instance the use of an improved vector-primed strategy (Petty *et al.*, 1988), of synthetic DNA "cassettes" (Rizzo and Palukaitis, 1990), of the polymerase chain reaction (PCR) for full-length (Hayes and Buck, 1990) or partial (Beck *et al.*, 1990; Macfarlane *et al.*, 1991; Young *et al.*, 1991; Boyer *et al.*, 1993; Demler *et al.*, 1993; Viry *et al.*, 1993) viral sequence amplification, and of "glass milk" for the isolation of a >9-kb-long first cDNA strand (Domier *et al.*, 1989). It is very likely that the production of full-length cDNAs from viral RNA templates will greatly benefit from the development of reverse transcriptases and DNA polymerases that are thermostable and endowed with increased fidelity.

Another problem is the high instability of full-length cDNA clones in bacteria. This is most strikingly exemplified by two related flaviviruses, yellow fever virus (YFV; Rice *et al.*, 1989) and Japanese encephalitis virus (JEV; Sumiyoshi *et al.*, 1992). In both cases, a full-length cDNA clone was successfully obtained. However, multiplication of this clone in bacteria always resulted in the introduction of mutations in the viral sequence. None of the corresponding *in vitro* transcripts was infectious. The only way to circumvent this problem and to synthesize infectious RNA was to perform *in vitro* transcription directly from *in vitro*-ligated fragments. This example might reflect the potential toxicity and/or instability of some viral sequences in bacteria, although the reasons for this are not clearly understood. It also provides a possible explanation of why no infectious transcripts have been obtained to date for some important viruses. Similar problems have been encountered for dengue virus type 4 (Lai *et al.*, 1991), RNA2 of beet necrotic yellow vein virus (BNYVV; Quillet *et al.*, 1989), pea early browning virus (PEBV; Macfarlane *et al.*, 1991), and eggplant mosaic virus (EMV; Skotnicki *et al.*, 1993). In some cases, these could be solved by changing the *Escherichia coli* strain and/or the DNA vector. Along the same line, the long homopolymeric tract of cytidyl residues (between 50 and 250 nucleotides) near the 5' end of the genomes of virus from the cardiovirus-aphtovirus group has for a long time hampered the construction of the corresponding full-length cDNA clones, possibly because long poly(dC-dG) tracts interfere with the replication of plasmids in *E. coli* (Deng and Wu, 1981). Shortening the length of the poly(C) tract in the case of mengovirus (Duke and Palmenberg, 1989) did not significantly affect the infectivity of the *in vitro* transcript, whereas a minimum of 32 Cs was required in the case of foot-and-mouth disease virus (FMDV; Zibert *et al.*, 1990).

An interesting result has been obtained with poliovirus by Kaplan *et al.* (1985). Since in the hands of these authors, no infectious transcripts containing a few nonviral nucleotides at either end were obtained,

³ Abbreviations used: AIMV, alfalfa mosaic virus; BBV, black beetle virus; BMV, brome mosaic virus; BNYVV, beet necrotic yellow vein virus; BSMV, barley stripe mosaic virus; BWYV, beet western yellows virus; BYDV, barley yellow dwarf virus; CaMV, cauliflower mosaic virus; CB3, Cocksackie B3 virus; CCMV, cowpea chlorotic mottle virus; CMV, cucumber mosaic virus; CNV, cucumber necrosis virus; CPMV, cowpea mosaic virus; CPSMV, cowpea severe mosaic virus; CYMV, clover yellow mosaic virus; CyRSV, cymbidium ringspot virus; EMV, eggplant mosaic virus; FMDV, foot-and-mouth disease virus; GFLV, grapevine fanleaf virus; HAV, hepatitis A virus; HRV, human rhinovirus; JEV, Japanese encephalitis virus; MCMV, maize chlorotic mottle virus; OYMV, onion yellow mosaic virus; PEBV, pea early browning virus; PEMV, pea enation mosaic virus; PMV, papaya mosaic virus; PPV, plum pox virus; PVX, potato virus X; RCNMV, red clover necrotic mosaic virus; RR, Ross river virus; SFV, Semliki forest virus; TEV, tobacco etch virus; TBSV, tomato bushy stunt virus; TCV, turnip crinkle virus; TMEV, Theiler's murine encephalomyelitis virus; TMV, tobacco mosaic virus; ToMV, tomato mosaic virus; TRV, tobacco rattle virus; TVMV, tobacco vein mottling; TYMV, turnip yellow mosaic virus; VEE, Venezuelan equine encephalitis virus; WCIMV, white clover mosaic virus; YFV, yellow fever virus; ZYMV, zucchini yellow mosaic virus; PCR, polymerase chain reaction; RdRp, RNA-dependent RNA polymerase; RNP, ribonucleoprotein.

another strategy was devised. Using SP6 RNA polymerase and the poliovirus cDNA-containing plasmid, negative strand poliovirus RNA was synthesized *in vitro*, which in turn served as template for the synthesis of plus strand RNA using the poliovirus RNA-dependent RNA polymerase (RdRp); the resulting plus strand RNA was infectious.

Infectious cDNAs

The expression of infectious viral RNAs through *in vivo* transcription of cDNA-containing vectors has several advantages. (1) Infectivity is less dependent on RNA degradation since it presumably occurs only within cells where the RNAs are synthesized, and the replication process can overcome detrimental effects resulting from degradation. (2) *In vitro* transcription is not necessary. This is particularly important for RNA viruses for which the production of a good yield of highly infectious full-length transcripts can be problematic. Furthermore, costly reagents such as the cap analogues and RNA polymerases are not required. (3) It renders the expression of viral genes largely independent of the viral replication process. This might be very convenient when studying the role and/or localization of proteins expressed by mutant viral RNAs unable to replicate in cells. These *in vivo*-produced viral transcripts would then behave like messenger RNAs produced by a host RNA polymerase, still able to express native or mutant proteins without being replicated (e.g., Van Bokhoven *et al.*, 1993).

Despite the fact that historically the first infectious clones of RNA viruses were cDNA clones, to date there are only a few examples of animal or plant viruses for which full-length cDNA-containing vectors were successfully used for the *in vivo* production of infectious RNA (Table 1A).

In certain cases, poliovirus cDNA was placed under the control of the SV40 promoter (Semler *et al.*, 1984; Kean *et al.*, 1986); it has, however, been postulated that the high specific infectivity of these cDNA clones would result from the presence of eukaryotic replication signals rather than from the effects of the promoter elements (Kean *et al.*, 1986).

A few recent examples exist for plant viruses that express infectious RNAs from *in vivo* transcription of viral cDNA-containing vectors. Infection of host plants was usually achieved by mechanical inoculation. Alternatively, infection was achieved through expression of infectious transcripts via transgenic plants (Yamaya *et al.*, 1988) or agroinfection (i.e., infection of plants with *Agrobacterium tumefaciens* expressing a viral RNA; Leiser *et al.*, 1992). All the corresponding viral cDNA sequences were placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter. In a few cases, unexplained differences in host susceptibility

have been observed between viral RNAs and the corresponding infectious cDNA constructs (Mori *et al.*, 1991; Weber *et al.*, 1992).

Despite the general necessity of RNA polymerase promoters for the efficient production of infectious transcripts from cDNA clones, several striking exceptions exist in which promoter sequences are not required or do not seem to be responsible for the synthesis of infectious RNAs. These examples include poliovirus (Racaniello and Baltimore, 1981; Omata *et al.*, 1984), Coxsackie B3 virus (CB3; Kandolf and Hofschneider, 1985), hepatitis A virus (HAV; Cohen *et al.*, 1987), and alfalfa mosaic virus (AIMV; RNA3 Dore and Pinck, 1988). The corresponding cDNAs were biologically active at relatively low levels. In addition, the mechanism whereby infectious RNAs are synthesized *in vivo* remains unclear. It has been proposed that the cDNA would somehow be transported to the nucleus, where it would be transcribed by a cellular DNA-dependent RNA polymerase initiating probably at "promoter-like" cryptic sites present on the DNA plasmid, or promoter activity could be provided by integration into the host genome, downstream of a cellular promoter (Racaniello and Baltimore, 1981; Omata *et al.*, 1984; Kandolf and Hofschneider, 1985; Klump *et al.*, 1990).

RNA polymerases: *In vitro* transcription

When dealing with the design of a full-length cDNA clone from which infectious RNAs are expected to be produced *in vitro* or *in vivo*, the choice of the RNA polymerase promoter is highly important because it directly affects the yield of transcripts and the nucleotide sequence at their extremities.

Several types of promoters have been used, such as the *E. coli* Pm (a modified version of Pr) promoter from bacteriophage λ , and the promoters of bacteriophages SP6, T3, or T7. The first transcription vector designed to provide exact control of the transcription initiation site was constructed by Ahlquist and Janda (1984). This vector, pPM1, allowed direct insertion into a unique *Sma*I cloning site of virtually any viral cDNA under the control of the phage λ Pm. Subsequent *in vitro* transcription with *E. coli* RNA polymerase produced transcripts bearing no extraviral nucleotides at their 5' end. The main advantage of this system resides in that in contrast to consensus promoter sequences for the T7 (Dunn and Studier, 1983) or SP6 (Melton *et al.*, 1984) RNA polymerases, that of *E. coli* lies well upstream of the initiation site for transcription and does not extend beyond the transcription initiation site (Hawley and McClure, 1983), allowing therefore virtually any λ Pm-viral cDNA fusion to be constructed without affecting promoter activity.

However, compared to phage RNA polymerase-based systems, the *E. coli* RNA polymerase-based

TABLE 1
INFECTIOUS CLONES FROM RNA VIRUSES

A						
FAMILY	VIRUS	RNA	LENGTH	PROM.	5'	3'
Leovirus	Q8	g	4.2	ND	ND	ND
Picornavirus	CB3	g	7.5	ND	ND	ND
	Polio	g	7.5	ND	ND	ND
	"	"	"	ND	ND	ND
	"	"	"	ND or SV40	ND	ND
Bromovirus	BMV	1,2,3	3.2; 2.9; 2.1	35S	12	ND
Comovirus	CPMV	B; M	5.9; 3.5	35S	0; 0	0; 7
Furovirus	BNYVV	3; 4	1.8; 1.5	35S	< 40	ND
Ilarivirus	AIMV	3	1.7	ND	ND	ND
"	"	1,2,3	3.6; 2.6; 2.1	35S	0	ND
"	"	g	4	0.9	35S	8
Luteovirus	BWYV	g	5.6	35S	0	ND
Potyvirus	YFV	g	9.7	35S	0	ND
Tobamovirus	TMV	g	6.4	35S	0	ND
"	ToMV	g	6.4	35S	0	ND
Tobravirus	PEBV	1,2	7.1; 3.4	35S	0	ND

B						
FAMILY	VIRUS	RNA	LENGTH	PROM.	5'	3'
Leovirus	Q8	g	4.2	T7	2	0
Alphavirus	RR	g	11.9	SP6; T7	1	ND
	SFV	g	11.4	SP6	0	4
	Sindbis	g	12	SP6	1	9
Flavivirus	YFV	g	9.7	T7	1	ND
	Dengue	g	10.6	SP6	1	5
	JEV	g	11	T7	0	0
Nodavirus	YFV	g	11	SP6	1	0
	BBV	1,2	3.1; 1.4	λPm	0	4
	"	g	"	SP6	20	4
Picornavirus	CB3	g	7.5	SP6; T7	82; 34	ND
	FMDV	g	8.1	SP6	12	ND
	HAV	g	7.5	SP6	10	0
	Mengo	g	7.8	T7	23	7
	Polio	g	7.5	T7	2	3-7
	"	g	"	T7	2	0
	HRV	g	7.5	SP6	21	2
	TMV	g	7.5	T7	2	5
Bromovirus	BMV	1,2,3	3.2; 2.8; 2.1	λPm	0	6-7
	"	"	"	T7	0	-1
	"	"	"	T7	0	7
Carmovirus	CCMV	1,2,3	3.2; 2.9; 2.1	T7	1	5
	TCV	g	4.0	λPm	1	5
	"	g	"	T7	2	5
Comovirus	CPMV	B; M	5.9; 3.5	T7	2	4-5
	"	"	"	T7	1	5
	"	M	3.5	λPm	0	7
Cucumovirus	CPSMV	1,2	6; 3.5	T7	1	25; 9
	CMV	1,2,3	3.4; 3.0; 2.1	T7	0	4
	"	"	"	T7	1	1
Dianthovirus	RCNMV	1,2	3.9; 1.4	T7	1	0
	PEMV	2	4.3	T7	0	0
	BNYVV	3; 4	1.8; 1.5	T7	1	12; 28
Hordeovirus	BSMV	α; β; γ	3.8; 3.3; 3.1	T7	0	< 4
	AIMV	3	2.1	T7	1	0
	"	4	0.9	SP6	15	2
Luteovirus	BYDV	g	5.7	T7	1	0
	BWYV	g	5.6	T7	2	6
	GFLV	1,2	7.3; 3.8	T7; T3	1	4; 24
Potyvirus	CYMV	g	7.0	T7	1	5
	PMV	g	6.7	T7	0	5
	PMV	g	6.4	T7	0	9
Potyvirus	WCIMV	g	5.8	SP6	0	6
	PPV	g	9.7	T7	1	12
	TEV	g	9.5	SP6	2	4
Tobravirus	TMV	g	9.5	T7	1	1
	"	g	"	T3	2	1
	ZYMV	g	9.6	T7	1	1
Sobemovirus	MCMV	g	4.4	T7	0	0
	TMV	g	6.4	λPm	0	3
	"	"	"	λPm	0	6
Tobamovirus	ToMV	g	6.4	T7	0	3
	PEBV	1,2	7.1; 3.4	T7	1-2	0
	TRV	1	6.8	λPm	0	0
Tombusvirus	CNV	g	4.7	T7	0	1
	CYRSV	g	4.7	T7	0	0
	TRSV	g	4.8	T7	2	0
Tymovirus	EMV	g	6.2	T7	0	3
	OYMV	g	6.3	T7	1	10
	TYMV	g	6.3	T7	0	5

system gives much lower yields of transcript, partly at least because it leads to a large proportion of premature termination products (Melton *et al.*, 1984; Janda *et al.*, 1987; Angenent *et al.*, 1989; Heaton *et al.*, 1989) and because important batch-to-batch variations of commercially available *E. coli* RNA polymerases strongly affect yield reproducibility (Janda *et al.*, 1987; Ahlquist *et al.*, 1987; Hamilton and Baulcombe, 1989).

The great majority of phage promoter-viral cDNA fusions involves the use of the T7 promoter, sometimes the SP6 promoter, and exceptionally the T3 promoter (Table 1B). Preference for the T7 over the SP6 promoter may be related to the more thoroughly studied genetics of bacteriophage T7.

Three different strategies have been used to fuse transcription promoters to the beginning of viral sequences. (1) "Universal" transcription vectors have been constructed in which a restriction site is inserted as close as possible to the transcription initiation site of the promoter. This is the case of the pM1/λPm (Ahlquist and Janda, 1984), pHST70/SP6 (Jobling *et al.*, 1988), and pCa35J/35S (Yamaya *et al.*, 1988) constructs. All these vectors could theoretically direct transcription of viral cDNA sequences into RNAs devoid of vector-derived nucleotides. (2) Extraneous sequences between the promoter and viral cDNA have been eliminated by site-directed mutagenesis. (3) Synthesis of second strand cDNA is primed with an oligonucleotide containing a promoter directly linked to the 5' end of the viral sequence. This procedure first described by Weiland and Dreher (1989) is now widely used for its simplicity and convenience.

For phage promoters, the "quality versus quantity" question has to be addressed in each individual case; the price to pay to obtain the most "viral-like" 5' end (supposedly necessary for good infectivity) is very often a low yield in transcription product, since the promoter-viral DNA fusions generally do not match optimum promoter consensus sequences.

The nucleotide sequence of the 3' end of *in vitro*-

Note to Table 1. (A) List of principal RNA viruses for which infectious cDNA clones have been obtained. (B) List of principal RNA viruses for which infectious *in vitro* transcripts have been obtained from full-length cDNA clones. For each virus, only the first and most significant works are mentioned.

* The SV40 promoter was introduced, but may not be the main determinant involved in the infectivity of the transcripts (Kean *et al.*, 1986). Prom., promoter; g, genomic; λPm, modified Pr promoter from phage λ; ND, not determined or not specified. Abbreviations of viruses are as listed under the Abbreviations used footnote. The RNA indicates the viral genome part for which the infectious cDNA or transcript has been obtained; g, genomic RNA in the case of viruses with a monopartite RNA genome. Length of the RNAs is in kilobases. 5' and 3' designate for each case the shortest nonviral extensions flanking the viral RNA sequence. The 3' values do not encompass the number of adenine residues in poly(A) tails.

transcribed full-length viral RNAs is commonly dictated by the position of the restriction site used for "run-off" transcription. However, Dzianott and Bujarski (1989) described an elegant alternative method to eliminate a large number of nonviral nucleotides at this extremity. This was achieved by ligating a self-processing sequence of the satellite RNA of tobacco ringspot virus to the 3' end of BMV sequences. The circular cDNA clones could be transcribed *in vitro* through a "rolling-circle"-like mechanism and the resulting multimeric transcripts were processed partially into infectious RNAs of genomic length. Since one has usually little control over transcription termination events in cells, this approach might be particularly useful for the processing of *in vivo*-transcribed RNAs which often harbor nonviral sequences such as vector-derived and/or poly(A) tails at their 3' end.

PARAMETERS AFFECTING INFECTIVITY

Infectious *in vitro*-synthesized transcripts

Although obtaining full-length cDNA clones and/or the corresponding transcripts is a crucial step, it does not necessarily ensure biological activity.

Relative infectivity of the infectious clones (Table 1) is very difficult to compare since the results obtained are expressed differently by various authors, depending on the system studied and on the method used. Indeed, infectivity can be looked at in terms of (1) the percentage of infected protoplasts (e.g., Loesch-Fries *et al.*, 1985) or systemic host plants showing symptoms (e.g., Hayes and Buck, 1990), (2) the relative number of local lesions on necrotic host plants for plant viruses (e.g., Heaton *et al.*, 1989) or the relative number of plaque-forming units for animal viruses (e.g., Kean *et al.*, 1986), and (3) the analysis of progeny viral products at the level of the RNA (e.g., Commandeur *et al.*, 1991) or proteins (e.g., Domier *et al.*, 1989). Infectivity of transcripts is very variable and can in some cases reach 100% or more (Sarnow, 1989; Hearne *et al.*, 1990; Hayes and Buck, 1990) compared to infectivity of wild-type virion RNAs. Moreover, since good transfection efficiencies help to detect low biological activities of some infectious clones, significant improvements have been recently achieved in this field, particularly for animal viruses (Keown *et al.*, 1990; Liljeström *et al.*, 1991).

To achieve successful infection, viral transcripts must interact with viral-encoded proteins, most particularly with the viral replicase and with host cell components such as the translation machinery; therefore, the structure of viral transcripts has to mimic that of virion RNA as closely as possible.

Several parameters have a dramatic influence on the infectivity of viral transcripts: (1) the heterogeneity of

transcript population, (2) the presence of point mutations, and (3) the sequence at the 5' and 3' ends (number and sequence of nonviral nucleotides, presence of a cap structure at the 5' end or a poly(A) tail at the 3' end).

The problem of heterogeneity of transcript size has been mainly reported with the *E. coli* RNA polymerase- λ Pm promoter system (Ahluquist *et al.*, 1984; Dawson *et al.*, 1986; Janda *et al.*, 1987; Hamilton and Baulcombe, 1989). A possible explanation for the relatively low infectivity of most preparations might be competition between incomplete nonreplicable viral copies and full-length transcripts for interaction with viral and/or host factors involved in the replication process.

Because of the relatively poor fidelity of the RNA- and DNA-synthesizing enzymes involved in producing infectious transcripts, point mutations are to be expected, especially with long viral genomes. For instance, alteration of viral sequences could result from the *in vitro* transcription step since sequence dependence of T7 and SP6 RNA polymerase fidelity has been reported (Kuhn *et al.*, 1990). Furthermore, the final full-length cDNA clone can be the result of faithful reverse transcription and amplification of an initial virion RNA which is itself mutated, unable to replicate, and which would most probably be eliminated in the next round of viral replication. As a consequence, this cDNA clone (or the corresponding *in vitro* transcript) would not be infectious. Given the low fidelity of viral RNA-dependent RNA polymerases (reviewed in Domingo and Holland, 1992; Ramirez *et al.*, 1993), this "unlucky" event might not be so rare. The production of noninfectious transcripts has been reported by a few authors (Ahluquist *et al.*, 1984; Kaplan *et al.*, 1985; Dawson *et al.*, 1986; Meshi *et al.*, 1986; Eggen *et al.*, 1989; Holt and Beachy, 1991; Lai *et al.*, 1991). In the last four cases, it has been possible to restore infectivity by exchanging a specific region of the cDNA with a fragment corresponding to the same region but issued from an independent cDNA clone. Conversely, certain synthetic transcripts induced more severe symptoms than the parental virion RNAs (Hamilton and Baulcombe, 1989; Hayes and Buck, 1990), thereby suggesting that possible mutations favoring infectivity of full-length transcripts might also occur.

Recent reports indicate that despite the high error rate of the *Taq* polymerase (Keohavong and Thilly, 1989), PCR can be successfully applied to obtain infectious clones. (Hayes and Buck, 1990; Viry *et al.*, 1993).

Since published sequences of viral RNA genomes do not necessarily correspond to infectious sequences, it can be instructive to determine the nucleotide sequence of infectious clones. This has been done in a few cases (Rice *et al.*, 1989; Beck *et al.*, 1990;

Hemenway *et al.*, 1990; Klump *et al.*, 1990; Dreher and Bransom, 1992).

Effect of nonviral nucleotides

The effect of nonviral nucleotides at the extremities of viral transcripts has been investigated by many authors. As a general rule, 5' extensions substantially decrease or even abolish infectivity, whereas 3' extensions are more easily tolerated.

Effect of 5' extensions. In most cases examined, infectivity is greatly diminished even for short 5' extensions such as 1 or 2 nucleotides (generally G residues).

Infectivity is abolished when transcripts derived from plant viruses harbor moderately long 5' additional sequences of 14 to 17 nucleotides (Heaton *et al.*, 1989; Dore *et al.*, 1990; Rizzo and Palukaitis, 1990). No infectious synthetic transcript presenting more than 6 nonviral residues at this extremity (Dawson *et al.*, 1986) has been described, with the exception of transcripts corresponding to AIMV RNA4 (Loesch-Fries *et al.*, 1985; Langereis *et al.*, 1986) bearing 15 additional bases at their 5' end. However, this latter exception could be related to the fact that translation of this RNA is sufficient to initiate infection when cotransfected with the other AIMV RNAs.

Commandeur *et al.* (1991) have recently shown for BNYVV that the cDNA sequences of RNAs 3 and 4 can be rendered biologically active when cloned downstream the CaMV 35S transcription promoter. The resulting *in vivo* transcripts expected to contain up to 40 extraviral nucleotides at the 5' end are infectious *in planta*, whereas *in vitro*-derived transcripts harboring such extensions are biologically inactive in the same host plant. Although these observations remain unexplained, they could illustrate the advantages of inducing transcription of viral genomes *in vivo* as discussed earlier.

Interestingly, compared to their plant RNA viruses counterparts, the infectivity of synthetic transcripts from several animal RNA viruses seems less affected when transcripts bear a relatively large number (10 to 82) of extraviral nucleotides at the 5' end (Mizutani and Colonno, 1985; Dasmahapatra *et al.*, 1986; Cohen *et al.*, 1987; Duke and Palmenberg, 1989; Klump *et al.*, 1990; Zibert *et al.*, 1990). Along the same line, several infectious cDNA clones of animal viruses (Table 1A) proved to be infectious despite the probable presence of large 5' extensions on the corresponding *in vivo* transcripts.

Although the number of cases reported is somehow limited and direct comparisons between plant and animal virus systems have to be taken with caution, it is questionable whether the differences observed in the effects of 5' extensions on infectivity of synthetic transcripts of some animal and plant viruses can reflect a

higher ability of host animal cell nucleases to "mature" *in vivo* the elongated transcripts into infectious RNAs, or a better tolerance in template recognition from RdRps of animal viruses.

The inhibitory effect of 5' nonviral nucleotides usually observed on the biological activity of synthetic viral transcripts could have several origins. Since the number of different extensions tested for various viral transcripts is limited in each case, it is difficult to deduce a role for the nucleotides in the nonviral sequences. It is also possible that the nature of the extra sequences is important, as suggested by the difference of infectivity observed between transcripts harboring nonviral 5' extensions similar in length but differing in their sequence (Mizutani and Colonno, 1985; Duechler *et al.*, 1989). However, it is usually assumed that the presence of these extra nucleotides could seriously hamper proper initiation of (+) RNA synthesis from the 3' end of the (-) strand. It seems unlikely that these nucleotides interfere with viral gene translation *in vivo* because they do not possess initiation codons, and the *in vitro* translation products are similar to those of wild-type RNA (Janda *et al.*, 1987; Verver *et al.*, 1987; Eggen *et al.*, 1989; Angenent *et al.*, 1989; Dore *et al.*, 1990; Gal-On *et al.*, 1991; Viry *et al.*, 1993). Several viruses commonly bear an additional nonviral encoded nucleotide at the 3' end of their (-) RNA strand, as do Sindbis virus (Wengler *et al.*, 1982) and cucumber mosaic virus (CMV; Rezaian *et al.*, 1984; Collmer and Kaper, 1985). This could be associated with the observation that full-length viral transcripts with only one nonviral nucleotide at their 5' end often show a relatively high level of infectivity, thereby possibly reflecting a certain level of tolerance in replicase/RNA sequence interaction.

Effect of 3' extensions. In contrast, the biological activity of viral transcripts appears to be relatively insensitive to short 3' extensions of 1 to 7 nucleotides (Dawson *et al.*, 1986; Ahlquist *et al.*, 1987; Eggen *et al.*, 1989; Dzianott and Bujarski, 1989; Hayes and Buck, 1990; Beck *et al.*, 1990), whereas long extensions such as 2700 nucleotides for brome mosaic virus (BMV, Ahlquist *et al.*, 1984), 945 nucleotides for TMV (Dawson *et al.*, 1986) or >82 nucleotides for BMV (Dzianott and Bujarski, 1989) abolish infectivity. For many viruses, however, transcripts bearing a long (>30 nucleotides) additional sequence at the 3' end are infectious (Dzianott and Bujarski, 1989; Eggen *et al.*, 1989; Beck *et al.*, 1990; Suzuki *et al.*, 1991; Boyer *et al.*, 1993; Sit and AbouHaidar, 1993), as are *in vivo* transcripts presumably polyadenylated by host cell enzymes. Sarnow (1989) specifically studied the effect of extraneous sequences at the 3' end of synthetic poliovirus transcripts and showed that while 4 extra bases (after a short poly(A) tract) did not lower the infectivity of the RNA, 17 cytosine residues decreased infectivity.

As is the case of 5' extensions, it is not clear whether

the structure of additional 3' sequences affects the biological activity of the transcripts. Interestingly, Dzianott and Bujarski (1989) reported that infectivity of BMV transcripts presenting 19 extraviral nucleotides was higher than those harboring 6 or 7 nonviral nucleotides at the 3' end. These authors speculate that a longer extension might confer protection *in vivo* against ribonucleases; alternatively, it could modify the secondary structure at the 3' end which would then acquire enhanced affinity for the BMV replicase. A similar observation has been made regarding CMV transcripts (Suzuki *et al.*, 1991). The observation that 3'-end-extended transcripts derived from plant viral genomes encompassing a tRNA-like structure are infectious strongly suggests that aminoacylation, presumably hampered on these transcripts, is not required for initial stages of viral infection or that host cell enzymes can process and/or degrade this extremity until it becomes aminoacylatable.

For viral genomes presenting a poly(A) tail at their 3' end, it seems probable that there is a threshold length of A residues below which the stability of the corresponding transcripts would be altered (Domier *et al.*, 1989; Sarnow, 1989; Hemenway *et al.*, 1990; Viry *et al.*, 1993; Holy and AbouHaidar, 1993). The presence of long homopolymeric adenine sequences at the 3' end of poliovirus *in vitro*-produced transcripts increases infectivity, whereas long heteropolymeric nucleotide sequences have an adverse effect (Sarnow, 1989). Eggen *et al.* (1989) have provided evidence that the poly(A) tail of cowpea mosaic virus (CPMV) transcripts is elongated upon replication in protoplasts. These authors hypothesize the possible involvement of a "slipping" RNA polymerase event or else the addition of A residues by a host terminal nucleotidyl transferase.

Effect of the cap structure. As a general rule, a cap structure (m⁷GpppG) is required at the 5' end of the transcripts for optimum infectivity, possibly because it enhances translation initiation (Shih *et al.*, 1976; Contreras *et al.*, 1982) and/or improves their stability by conferring a better resistance to host cell nucleases (Furuichi *et al.*, 1977; Shimotohno *et al.*, 1977; Green *et al.*, 1983).

Uncapped transcripts are either not infectious (Ahlquist *et al.*, 1984; Loesch-Fries *et al.*, 1985; Dawson *et al.*, 1986; Petty *et al.*, 1989; Weiland and Dreher, 1989; Domier *et al.*, 1989; Riechmann *et al.*, 1990; Gal-On *et al.*, 1991; Kuhn *et al.*, 1991; Boyer *et al.*, 1993) or show a highly reduced level of infectivity (Dasmahapatra *et al.*, 1986; Meshi *et al.*, 1986; Janda *et al.*, 1987; Rice *et al.*, 1987; Allison *et al.*, 1988; Vos *et al.*, 1988; Eggen *et al.*, 1989; Hamilton and Baulcombe, 1989; Beck *et al.*, 1990; Hayes and Buck, 1990; Liljeström *et al.*, 1991; Xiong and Lommel, 1991; Veidt *et al.*, 1992; Sit and Abouhaidar, 1993). However, in a few cases, both

capped and uncapped transcripts proved to be highly infectious (Angenent *et al.*, 1989; Heaton *et al.*, 1989; Hearne *et al.*, 1990; Rochon and Johnston, 1991; Scheets *et al.*, 1993).

A striking example is that of tobacco rattle virus (TRV) transcripts. Whereas TRV RNA1 transcripts require the presence of a capped 5' end for infectivity (Hamilton and Baulcombe, 1989), TRV RNA2 transcripts do not (Angenent *et al.*, 1989). As postulated (Angenent *et al.*, 1989), it is possible that efficient synthesis of the TRV replicase requires capped TRV RNA1 transcripts. The replicase would then accept uncapped RNA2 transcripts as template to generate sub-genomic coat protein messenger RNA and progeny RNA2. A similar situation has been reported for transcripts from the related PEBV (MacFarlane *et al.*, 1991) and for BMV transcripts (Ahlquist *et al.*, 1987).

Many RNA viruses harbor a viral-encoded protein (VPg) instead of a cap structure at the 5' extremity of their genome. Studies performed with the corresponding *in vitro* transcripts demonstrated in most cases the positive effect of capping on their infectivity (although at various levels; e.g., Eggen *et al.*, 1989; Riechmann *et al.*, 1990; Young *et al.*, 1991; Veidt *et al.*, 1992), suggesting that some of the properties of the cap structure mentioned earlier could at least partially compensate the lack of VPg. It is likely that studies on the role of VPg in infectivity will greatly benefit from an efficient method for adding this protein to synthetic transcripts. Along this line, the RNA-catalyzed *in vitro* addition of VPg on (–)RNA of poliovirus has been reported (Tobin *et al.*, 1989) and might open new perspectives in this field.

Fate of nonviral nucleotides in virus progeny

In all cases where this has been verified by sequencing of progeny RNAs or by molecular hybridization, extraviral 5' or 3' sequences are removed in cells upon viral replication or restored to wild-type length (Ahlquist *et al.*, 1984; Dasmahapatra *et al.*, 1986; Meshi *et al.*, 1986; Ziegler-Graff *et al.*, 1988; Duke and Palmenberg, 1989; Dzianott and Bujarski, 1989; Eggen *et al.*, 1989; Heaton *et al.*, 1989; Quillet *et al.*, 1989; Beck *et al.*, 1990; Hearne *et al.*, 1990; Klump *et al.*, 1990; Riechmann *et al.*, 1990; Commandeur *et al.*, 1991; Mori *et al.*, 1991; Viry *et al.*, 1993). As mentioned earlier, host cell nucleases could participate in eliminating nonviral extensions, thereby rendering the transcript more amenable to enter replication cycles. Alternatively, viral RdRps would be able to initiate replication of elongated transcripts by recognizing their cognate binding site internally, which would lead to the elimination of extra sequences in the daughter strand. In the case of *in vivo* transcripts, inaccurate initiation of transcription by host RNA polymerases could also lead to the same

result by favoring the direct and fortuitous production of suitable templates for viral RdRps.

Interestingly, the production of infectious transcripts missing terminal residues has also been reported: CB3 transcripts lacking the two 5' uridine residues were found to be infectious and to restore these nucleotides upon replication in transfected cells, suggesting that VPg-pUpU may be uridylylated through a template-independent process (Klump *et al.*, 1990), and BMV transcripts lacking the last A residue at the 3' end were highly infectious, most likely because the host ATP, CTP: tRNA nucleotidyltransferase can restore the missing nucleotide *in vivo* (Ahlquist *et al.*, 1987).

In contrast to terminal extensions, more internal point mutations introduced accidentally or as "molecular labels" are often maintained in progeny RNAs (Meshi *et al.*, 1986; Hearne *et al.*, 1990; Zibert *et al.*, 1990; Lai *et al.*, 1991; Weber *et al.*, 1992). However, this observation reflects probably more the nonlethal character of these internal mutations than the inability of the system to correct them.

The fate of longer internal modifications seems less predictable. Indeed, insertion of nonviral genes and artificial elongation or reduction of internal oligo(A) regions and poly(C) tracts were shown to lead to either situation in progeny RNAs: maintenance (Duke and Palmenberg, 1989; Dolja *et al.*, 1992) or modification (Allison *et al.*, 1988; Zibert *et al.*, 1990; Dolja *et al.*, 1992) of these regions toward wild-type length or sequence.

Negative-strand RNA viruses

As opposed to the tremendous progress made over the past decade on the construction of infectious cDNA clones and transcripts of positive strand RNA viruses, such experiments have lagged behind with negative strand RNA viruses. This is because with the latter viruses naked genomic RNA introduced into cells or synthesized intracellularly from transfected cDNA clones are not "infectious" *per se* since they cannot be replicated in the absence of viral nucleoprotein and polymerase. This situation dramatically changed with the work of Luytjes *et al.* (1989) using *in vitro* transcripts containing the regulatory sequences of one of the influenza virus genomic RNAs. Biologically active ribonucleoprotein (RNP) complexes were reconstituted *in vitro* using these synthetic RNAs, together with purified viral nucleoprotein and polymerase proteins. In the form of RNPs, these RNAs could then be amplified, expressed, and packaged when introduced into cells with helper virus (Luytjes *et al.*, 1989). This "rescue" approach was also successfully used for nonsegmented negative strand RNA viruses (Park *et al.*, 1991; Collins *et al.*, 1991), which have large genomes (12–15 kb) less amenable to cloning technology. An alternative

strategy has been to transfect simultaneously nucleocapsids or cloned viral cDNAs (usually corresponding to artificial or natural defective interfering genomes interrupted by a reporter gene) with a set of T7- and/or vaccinia virus-based cDNA vectors expressing viral proteins (e.g., Fuerst *et al.*, 1986; Pattnaik and Wertz, 1990; Curran *et al.*, 1991; Jin and Elliott, 1991; Calain *et al.*, 1992; Pattnaik *et al.*, 1992).

In all cases, however, the transcripts studied are not of genomic size since they contain mainly terminal regulatory regions bordering (or not) a reporter gene and they still have to be complemented *in trans* with viral proteins for biological activity. Nevertheless, using the systems mentioned above and despite the absence of infectious synthetic clones, excellent progress has been made these last years in deciphering the role of *cis*- and *trans*-acting factors involved in transcription, replication, and assembly of these viruses.

CONCLUSIONS

To date, infectious *in vitro* transcripts or cDNA clones have been obtained for a wide number of unrelated RNA viruses. Strikingly, the majority are plant viruses, despite the fact that the first publication of this kind dealt with bacteriophage Q β (Taniguchi *et al.*, 1978) and the second with an animal virus (Racaniello and Baltimore, 1981). It is also interesting that there exist only a few reports of the expression of infectious RNAs via an *in vivo* expression vector as opposed to reports concerning the production of infectious transcripts obtained *in vitro*.

Despite the many advantages provided by highly infectious clones, it is important to bear in mind that these clones might present a potential hazard *in vivo* for the following reasons: (1) in some reported experiments, transcripts appear to be more infectious than the corresponding wild-type virus; (2) since the sequence of infectious clones has only been determined in a few cases, it is usually assumed, but not demonstrated, that this sequence is identical to that of the virus and will behave as such *in vivo*; (3) infectious transcripts are biologically amplifiable synthetic molecules, and like their wild-type viral RNA counterpart, they are subject to RNA recombination events and/or genetic modifications resulting from the viral replication process (e.g., point mutations and template-switching activity from the error-prone viral replicase). Therefore, it seems reasonable to restrict the utilization of infectious clones to confined and controlled biological environments.

By rendering viral genetic information more accessible to targeted alterations and modifications, by enabling pure viral genomes to be obtained through cDNA cloning techniques, infectious clones have very quickly become invaluable and powerful tools for mo-

lecular virologists and will probably help in deciphering crucial problems addressed by the complexity of the parameters involved in the infection process mediated by RNA viruses.

ACKNOWLEDGMENTS

We are most grateful to Dr. S. van der Werf, J. Wellink and R. Kormelink for helpful comments on the manuscript. This research was supported in part by a grant from the "Action Incitative Programmée: Plantes transgéniques résistantes aux virus." It was also supported by an anonymous gift, a grant from the "Ligue Nationale Française Contre le Cancer" and then a FEBS long-term fellowship to J.-C.B.

REFERENCES

- AHLQUIST, P., and JANDA, M. (1984). cDNA cloning and *in vitro* transcription of the complete brome mosaic virus genome. *Mol. Cell. Biol.* **4**, 2876–2882.
- AHLQUIST, P., FRENCH, R., JANDA, M., and LOESCH-FRIES, L. S. (1984). Multicomponent RNA plant virus infection derived from cloned viral cDNA. *Proc. Natl. Acad. Sci. USA* **81**, 7066–7070.
- AHLQUIST, P., FRENCH, R., and BUJARSKI, J. J. (1987). Molecular studies of brome mosaic virus using infectious transcripts from cloned cDNA. *Adv. Virus Res.* **32**, 215–242.
- ALLISON, R. F., JANDA, M., and AHLQUIST, P. (1988). Infectious *in vitro* transcripts from cowpea chlorotic mottle virus cDNA clones and exchange of individual RNA components with brome mosaic virus. *J. Virol.* **62**, 3581–3588.
- ANGENENT, G. C., POSTHUMUS, E., and BOL, J. F. (1989). Biological activity of transcripts synthesized *in vitro* from full-length and mutated DNA copies of tobacco rattle virus RNA 2. *Virology* **173**, 68–76.
- BECK, D. L., FORSTER, R. L. S., BEVAN, M. W., BOXEN, K. A., and LOWE, S. C. (1990). Infectious transcripts and nucleotide sequence of cloned cDNA of the potyvirus white clover mosaic virus. *Virology* **177**, 152–158.
- BOYER, J.-C., DRUGÉON, G., SERON, K., MORCH-DEVIGNES, M.-D., AGNES, F., and HAENNI, A.-L. (1993). *In vitro* transcripts of turnip yellow mosaic virus encompassing a long 3' extension or produced from a full-length cDNA clone harboring a 2-kb-long PCR-amplified segment are infectious. *Res. Virol.* **144**, 339–348.
- BUJARSKI, J. J., and MILLER, W. A. (1992). Use of *in vitro* transcription to study gene expression and replication of spherical, positive sense RNA plant viruses. In "Genetic Engineering with Plant Viruses" (T. M. A. Wilson and J. W. Davies, Eds.), pp. 115–147. CRC Press, Boca Raton, FL.
- BURGYAN, J., NAGY, P. D., and RUSSO, M. (1990). Synthesis of infectious RNA from full-length cloned cDNA to RNA of cymbidium ringspot tomosvirus. *J. Gen. Virol.* **71**, 1857–1860.
- CALAIN, P., CURRAN, J., KOLAKOSKY, D., and ROUX, L. (1992). Molecular cloning of natural paramyxovirus copy-back defective interfering RNAs and their expression from DNA. *Virology* **191**, 62–71.
- CHEN, X., and BRUENING, G. (1992). Cloned DNA copies of cowpea severe mosaic virus genomic RNAs—Infectious transcripts and complete nucleotide sequence of RNA-1. *Virology* **191**, 607–618.
- COHEN, J. I., TICEHURST, J. R., FEINSTONE, S. M., ROSENBLUM, B., and PURCELL, R. H. (1987). Hepatitis A virus cDNA and its RNA transcripts are infectious in cell culture. *J. Virol.* **61**, 3035–3039.
- COLLINS, P. L., MINK, M. A., and STEC, D. S. (1991). Rescue of synthetic analogs of respiratory syncytial virus genomic RNA and effect of truncations and mutations on the expression of a foreign reporter gene. *Proc. Natl. Acad. Sci. USA* **88**, 9663–9667.
- COLLMER, C. W., and KAPER, J. M. (1985). Double-stranded RNAs of CMV and its satellite contain an unpaired terminal guanosine: Implication for replication. *Virology* **145**, 249–253.
- COMMANDEUR, U., JARAUSCH, W., LI, Y., KOENIG, R., and BURGERMEISTER, W. (1991). cDNAs of beet necrotic yellow vein virus RNA-3 and RNA-4 are rendered biologically active in a plasmid containing the cauliflower mosaic virus 35S promoter. *Virology* **185**, 493–495.
- CONTRERAS, R., CHEROUTRE, H., DEGRAVE, W., and FIER, W. (1982). Simple, efficient *in vitro* synthesis of capped RNA useful for direct expression of cloned eukaryotic genes. *Nucleic Acids Res.* **10**, 6353–6362.
- CURRAN, J., BOECK, R., and KOLAKOSKY, D. (1991). The Sendai virus P gene expresses both an essential protein and an inhibitor of RNA synthesis by shuffling modules via mRNA editing. *EMBO J.* **10**, 3079–3085.
- DASMAHAPATRA, B., DASGUPTA, R., SAUNDERS, K., SELLING, B., GALLAGHER, T., and KAESBERG, P. (1986). Infectious RNA derived by transcription from cloned cDNA copies of the genomic RNA of an insect virus. *Proc. Natl. Acad. Sci. USA* **83**, 63–66.
- DAVIS, N. L., WILLIS, L. V., SMITH, J. F., and JOHNSTON, R. E. (1989). *In vitro* synthesis of infectious Venezuelan equine encephalitis virus RNA from a cDNA clone: Analysis of a viable deletion mutant. *Virology* **171**, 189–204.
- DAWSON, W. O., BECK, D. L., KNORR, D. A., and GRANTHAM, G. L. (1986). cDNA cloning of the complete genome of tobacco mosaic virus and production of infectious transcripts. *Proc. Natl. Acad. Sci. USA* **83**, 1832–1836.
- DEMLER, S. A., RUCKER, D. G., and DE ZOETEN, G. A. (1993). The chimeric nature of the genome of pea enation mosaic virus: The independent replication of RNA2. *J. Gen. Virol.* **74**, 1–14.
- DENG, G., and WU, R. (1981). An improved procedure for utilizing terminal transferase to add homopolymers to the 3' termini of DNA. *Nucleic Acids Res.* **9**, 4173–4188.
- DESSENS, J. T., and LOMONOSOFF, G. P. (1993). Cauliflower mosaic virus 35S promoter-controlled DNA copies of cowpea mosaic virus RNAs are infectious on plants. *J. Gen. Virol.* **74**, 889–892.
- DOLJA, V. V., MCBRIDE, H. J., and CARRINGTON, J. C. (1992). Tagging of plant potyvirus replication and movement by insertion of β -glucuronidase into the viral polyprotein. *Proc. Natl. Acad. Sci. USA* **89**, 10208–10212.
- DOMIER, L. L., FRANKLIN, K. M., HUNT, A. G., RHOADS, R. E., and SHAW, J. G. (1989). Infectious *in vitro* transcripts from cloned cDNA of a potyvirus, tobacco vein mottling virus. *Proc. Natl. Acad. Sci. USA* **86**, 3509–3513.
- DOMINGO, E., and HOLLAND, J. J. (1992). Mutation rates and rapid evolution of RNA viruses. In "Evolutionary Biology of Viruses" (S. S. Morse, Ed.). Raven Press.
- DORE, J.-M., and PINCK, L. (1988). Plasmid DNA containing a copy of RNA3 can substitute for RNA3 in alfalfa mosaic virus RNA inocula. *J. Gen. Virol.* **69**, 1331–1338.
- DORE, J.-M., ERNY, C., and PINCK, L. (1990). Biologically active transcripts of alfalfa mosaic virus RNA3. *FEBS Lett.* **264**, 183–186.
- DREHER, T. W., and BRANSOM, K. L. (1992). Genomic RNA sequence of turnip yellow mosaic virus isolate TYMC, a cDNA-based clone with verified infectivity. *Plant Mol. Biol.* **18**, 403–406.
- DUECHLER, M., SKERN, T., BLAAS, D., BERGER, B., SOMMERGRUBER, W., and KUECHLER, E. (1989). Human rhinovirus serotype 2: *In vitro* synthesis of an infectious RNA. *Virology* **168**, 159–161.
- DUKE, G. M., and PALMENBERG, A. C. (1989). Cloning and synthesis of infectious cardiobvirus RNAs containing short, discrete poly(C) tracts. *J. Virol.* **63**, 1822–1826.
- DUNN, J. J., and STUDIER, F. W. (1983). Complete nucleotide sequence of bacteriophage T7 DNA and the location of T7 genetic elements. *J. Mol. Biol.* **166**, 477–485.
- DZIANOTT, A. M., and BUJARSKI, J. J. (1989). Derivation of an infectious viral RNA by autocatalytic cleavage of *in vitro* transcribed viral cDNAs. *Proc. Natl. Acad. Sci. USA* **86**, 4823–4827.

- EGGEN, R., VERVER, J., WELLINK, J., DE JONG, A., GOLDBACH, R., and VAN KAMMEN, A. (1989). Improvements of the infectivity of *in vitro* transcripts from cloned cowpea mosaic virus cDNA: Impact of terminal nucleotide sequences. *Virology* **173**, 447-455.
- FRANKEL, G., and FRIEDMANN, A. (1987). Synthesis of long viral complementary DNA from 7.5 kb poly A + RNA templates. *J. Virol. Methods* **18**, 1-12.
- FUERST, T. R., NILES, E. G., STUDIER, F. W., and MOSS, B. (1986). Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA* **83**, 8122-8126.
- FURUICHI, Y., LAFIANDRA, A., and SHATKIN, A. J. (1977). 5'-Terminal structure and mRNA stability. *Nature* **266**, 235-239.
- GAL-ON, A., ANTIGNUS, Y., ROSNER, A., and RACCAH, B. (1991). Infectious *in vitro* RNA transcripts derived from cloned cDNA of the cucurbit potyvirus, zucchini yellow mosaic virus. *J. Gen. Virol.* **72**, 2639-2643.
- GREEN, M. R., MANIATIS, T., and MELTON, D. A. (1983). Human β -globin pre-mRNA synthesized *in vitro* is accurately spliced in *Xenopus* oocyte nuclei. *Cell* **32**, 681-694.
- GUBLER, U., and HOFFMAN, B. J. (1983). A simple and very efficient method for generating cDNA libraries. *Gene* **25**, 263-269.
- HAMILTON, W. D. O., and BAULCOMBE, D. C. (1989). Infectious RNA produced by *in vitro* transcription of a full-length tobacco rattle virus RNA-1 cDNA. *J. Gen. Virol.* **70**, 963-968.
- HAWLEY, D., and MCCLURE, W. (1983). Compilation and analysis of *Escherichia coli* promoter DNA sequences. *Nucleic Acids Res.* **11**, 2237-2255.
- HAYES, R. J., and BUCK, K. W. (1990). Infectious cucumber mosaic virus RNA transcribed *in vitro* from clones obtained from cDNA amplified using the polymerase chain reaction. *J. Gen. Virol.* **71**, 2503-2508.
- HEARNE, P. Q., KNORR, D. A., HILLMAN, B. I., and MORRIS, T. J. (1990). The complete genome structure and synthesis of infectious RNA from clones of tomato bushy stunt virus. *Virology* **177**, 141-151.
- HEATON, L. A., CARRINGTON, J. C., and MORRIS, T. J. (1989). Turnip crinkle virus infection from RNA synthesized *in vitro*. *Virology* **170**, 214-218.
- HEMENWAY, C., WEISS, J., O'CONNELL, K., and TUMER, N. E. (1990). Characterization of infectious transcripts of a potato virus X clone. *Virology* **175**, 365-371.
- HOLNESS, C. L., LOMONOSOFF, G. P., EVANS, D., and MAULE, A. J. (1989). Identification of the initiation codons for translation of cowpea mosaic virus middle component RNA using site-directed mutagenesis of an infectious cDNA clone. *Virology* **172**, 311-320.
- HOLT, C. A., and BEACHY, R. N. (1991). *In vivo* complementation of infectious transcripts from mutant tobacco mosaic virus cDNAs in transgenic plants. *Virology* **181**, 109-117.
- HOLY, S., and ABOUHAIKAR, M. G. (1993). Production of infectious *in vitro* transcripts from a full-length clover yellow mosaic virus cDNA clone. *J. Gen. Virol.* **74**, 781-784.
- JANDA, M., FRENCH, R., and AHLQUIST, P. (1987). High efficiency T7 polymerase synthesis of infectious RNA from cloned brome mosaic virus cDNA and effects of 5' extensions on transcript infectivity. *Virology* **158**, 259-262.
- JIN, H., and ELLIOTT, R. M. (1991). Expression of functional bunyamwera virus L protein by recombinant vaccinia virus. *J. Virol.* **65**, 4182-4189.
- JOBLING, S. A., CUTHBERT, C. M., ROGERS, S. G., FRALEY, R. T., and GEHRKE, L. (1988). *In vitro* transcription and translational efficiency of chimeric SP6 messenger RNAs devoid of 5' vector nucleotides. *Nucleic Acids Res.* **10**, 4483-4498.
- KANDOLF, R., and HOFSCHEIDER, P. H. (1985). Molecular cloning of the genome of a cardiotropic coxsackie B3 virus: Full-length reverse transcribed recombinant cDNA generates infectious virus in mammalian cells. *Proc. Natl. Acad. Sci. USA* **82**, 4818-4822.
- KAPLAN, G., LUBINSKI, J., DASGUPTA, A., and RACANIELLO, V. R. (1985). *In vitro* synthesis of infectious poliovirus RNA. *Proc. Natl. Acad. Sci. USA* **82**, 8424-8428.
- KEAN, K. M., WYCHOWSKI, C., KOPECKA, H., and GIRARD, M. (1986). Highly infectious plasmids carrying poliovirus cDNA are capable of replication in transfected simian cells. *J. Virol.* **59**, 490-493.
- KEOHAVONG, P., and THILLY, W. G. (1989). Fidelity of DNA polymerases in DNA amplification. *Proc. Natl. Acad. Sci. USA* **86**, 9253-9257.
- KEOWN, W. A., CAMPBELL, C. R., and KUCHERLAPATI, R. S. (1990). Methods for introducing DNA into mammalian cells. *Methods Enzymol.* **185**, 537-537.
- KLUMP, W. M., BERGMANN, I., MÜLLER, B. C., AMEIS, D., and KANDOLF, R. (1990). Complete nucleotide sequence of infectious coxsackievirus B3 cDNA: Two initial 5' uridine residues are regained during plus-strand RNA synthesis. *J. Virol.* **64**, 1573-1583.
- KUHN, R., HONG, Z., and STRAUSS, J. H. (1990). Mutagenesis of the 3' nontranslated region of Sindbis virus RNA. *J. Virol.* **64**, 1465-1476.
- KUHN, R. J., NIESTERS, H. G. M., HONG, Z., and STRAUSS, J. H. (1991). Infectious RNA transcripts from Ross river virus cDNA clones and the construction and characterization of defined chimeras with sindbis virus. *Virology* **182**, 430-441.
- LAI, C. J., ZHAO, B., HORI, H., and BRAY, M. (1991). Infectious RNA transcribed from stably cloned full-length cDNA of dengue type-4 virus. *Proc. Natl. Acad. Sci. USA* **88**, 5139-5143.
- LANGEREIS, K., NEELEMAN, L., and BOL, J. F. (1986). Biologically active transcripts of cloned DNA of the coat protein messenger of two plant viruses. *Plant Mol. Biol.* **6**, 281-288.
- LEISER, R.-M., ZIEGLER-GRAFF, V., REUTENAUER, A., HERRBACH, E., LE-MAIRE, O., GUILLEY, H., RICHARDS, K., and JONARD, G. (1992). Agroinfection as an alternative to insects for infecting plants with beet western yellows luteovirus. *Proc. Natl. Acad. Sci. USA* **89**, 9136-9140.
- LILJESTRÖM, P., LUSA, S., HUYLEBROECK, D., and GAROFF, H. (1991). *In vitro* mutagenesis of a full-length cDNA clone of Semliki forest virus: The small 6,000-molecular-weight membrane protein modulates virus release. *J. Virol.* **65**, 4107-4113.
- LOESCH-FRIES, L. S., JARVIS, N. P., KRAHN, K. J., NELSON, S. E., and HALL, T. C. (1985). Expression of alfalfa mosaic virus RNA 4 cDNA transcripts *in vitro* and *in vivo*. *Virology* **146**, 177-187.
- LUYTJES, W., KRISTAL, M., ENAMI, M., PARVIN, J. D., and PALESE, P. (1989). Amplification, expression, and packaging of a foreign gene by influenza virus. *Cell* **59**, 1107-1113.
- MACFARLANE, S. A., WALLIS, C. V., TAYLOR, S. C., GOULDEN, M. G., WOOD, K. R., and DAVIES, J. W. (1991). Construction and analysis of infectious transcripts synthesized from full-length clones of both genomic RNAs of pea early browning virus. *Virology* **182**, 124-129.
- MACFARLANE, S. A., GILMER, D., and DAVIES, J. W. (1992). Efficient inoculation with CaMV 35S promoter-driven DNA clones of the tobamovirus PEBV. *Virology* **187**, 829-831.
- MAISS, E., TIMPE, U., BRISSEKRODE, A., LESEMANN, D. E., and CASPER, R. (1992). Infectious *in vivo* transcripts of a plum pox potyvirus full-length cDNA clone containing the cauliflower mosaic virus-35S RNA promoter. *J. Gen. Virol.* **73**, 709-713.
- MELTON, D. A., KRIEG, P. A., REBAGLIATI, M. R., MANIATIS, T., ZINN, K., and GREEN, M. R. (1984). Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* **12**, 7035-7056.
- MESHI, T., ISHIKAWA, M., MOTOYOSHI, F., SEMBA, K., and OKADA, Y. (1986). *In vitro* transcription of infectious RNAs from full-length cDNAs of tobacco mosaic virus. *Proc. Natl. Acad. Sci. USA* **81**, 1966-1970.
- MIZUTANI, S., and COLONNO, R. J. (1985). *In vitro* synthesis of an infectious RNA from cDNA clones of human rhinovirus type 14. *J. Virol.* **56**, 628-632.

- MORI, M., MISE, K., KOBAYASHI, K., OKUNO, T., and FUROSAWA, I. (1991). Infectivity of plasmids containing brome mosaic virus cDNA linked to the cauliflower mosaic virus 35S RNA promoter. *J. Gen. Virol.* **72**, 243–246.
- NEELEMAN, L., VAN DER VOSSEN, E. A. G., and BOL, J. F. (1993). Infection of tobacco with alfalfa mosaic virus cDNAs sheds light on the early function of the coat protein. *Virology* **196**, 883–887.
- OKAYAMA, H., and BERG, P. (1982). High-efficiency cloning of full-length cDNA. *Mol. Cell. Biol.* **2**, 161–170.
- OMATA, T., KOHARA, M., SAKAI, Y., KAMEDA, A., IMURA, N., and NOMOTO, A. (1984). Cloned infectious complementary DNA of the poliovirus Sabin 1 genome: Biochemical and biological properties of recovered virus. *Gene* **32**, 1–10.
- PARK, K. H., HUANG, T., CORREIA, F. F., and KRISTAL, M. (1991). Rescue of a foreign gene by Sendai virus. *Proc. Natl. Acad. Sci. USA* **88**, 5537–5541.
- PATTNAIK, A. K., and WERTZ, G. W. (1990). Replication and amplification of defective interfering particle RNAs of vesicular stomatitis virus in cells expressing viral proteins from vectors containing cloned cDNAs. *J. Virol.* **64**, 2948–2957.
- PATTNAIK, A. K., BALL, L. A., LEGRONE, A. W., and WERTZ, G. W. (1992). Infectious defective interfering particles of VSV from transcripts of a cDNA clone. *Cell* **69**, 1011–1020.
- PETTY, I. T. D., HUNTER, B. G., and JACKSON, A. O. (1988). A novel strategy for one-step cloning of full-length cDNA and its application to the genome of barley stripe mosaic virus. *Gene* **74**, 423–432.
- PETTY, I. T. D., HUNTER, B. G., WEI, N., and JACKSON, A. O. (1989). Infectious barley stripe mosaic virus RNA transcribed *in vitro* from full-length genomic cDNA clones. *Virology* **171**, 342–349.
- QUILLET, L., GUILLEY, H., JONARD, G., and RICHARDS, K. (1989). *In vitro* synthesis of biologically active beet necrotic yellow vein virus RNA. *Virology* **172**, 293–301.
- RACANELLO, V. R., and BALTIMORE, D. (1981). Cloned poliovirus cDNA is infectious in mammalian cells. *Science* **214**, 916–919.
- RAMIREZ, B. C., BARBIER, P., SERON, K., HAENNI, A.-L., and BERNARDI, F. (1993). Molecular mechanisms of point mutations in RNA viruses. In *"Molecular Basis of Viral Evolution"* (Gibbs and Calisher, Eds.), Cambridge Univ. Press, in press.
- REZAIAN, M. A., WILLIAMS, R. H. V., GORDON, K. H. J., GOULD, A. R., and SYMONS, R. H. (1984). Nucleotide sequence of cucumber mosaic virus RNA 2 reveals a translation product significantly homologous to corresponding proteins of other viruses. *Eur. J. Biochem.* **143**, 277–281.
- RICE, C. M., LEVIS, R., STRAUSS, J. H., and HUANG, H. V. (1987). Production of infectious RNA transcripts from sindbis virus cDNA clones: Mapping of lethal mutations, rescue of a temperature-sensitive marker, and *in vitro* mutagenesis to generate defined mutants. *J. Virol.* **61**, 3809–3819.
- RICE, C. M., GRAKOU, A., GALLER, R., and CHAMBERS, T. J. (1989). Transcription of infectious yellow fever RNA from full-length cDNA templates produced by *in vitro* ligation. *New Biol.* **1**, 285–296.
- RIECHMANN, J. L., LAIN, S., and GARCIA, J. A. (1990). Infectious *in vitro* transcripts from plum Pox potyvirus cDNA clone. *Virology* **177**, 710–716.
- RIZZO, T. M., and PALUKAITIS, P. (1990). Construction of full-length cDNA clones of cucumber mosaic virus RNAs 1, 2 and 3: Generation of infectious transcripts. *Mol. Gen. Genet.* **222**, 249–256.
- ROCHON, D. M., and JOHNSTON, J. C. (1991). Infectious transcripts from cloned cucumber necrosis virus cDNA—Evidence for a bi-functional subgenomic mRNA. *Virology* **181**, 656–665.
- ROOS, R. P., STEIN, S., OHARA, Y., FU, J., and SEMLER, B. L. (1989). Infectious cDNA clones of the DA strain of Theiler's murine encephalomyelitis virus. *J. Virol.* **63**, 5492–5496.
- SARNOW, P. (1989). Role of 3'-end sequences in infectivity of poliovirus transcripts made *in vitro*. *J. Virol.* **63**, 467–470.
- SCHETS, K., KHOSRAVI-FAR, R., and NUTTER, R. C. (1993). Transcripts of a maize chlorotic mottle virus cDNA clone replicate in maize protoplasts and infect maize plants. *Virology* **193**, 1006–1009.
- SCHMID, A., CATTANEO, R., and BILLETER, M. A. (1987). A procedure for selective full-length cDNA cloning of specific RNA species. *Nucleic Acids Res.* **15**, 3987–3996.
- SEMLER, B. L., DORNER, A. J., and WIMMER, E. (1984). Production of infectious poliovirus from cloned cDNA is dramatically increased by SV40 transcription and replication signals. *Nucleic Acids Res.* **12**, 5123–5141.
- SHIH, D. S., DASGUPTA, R., and KAESBERG, P. (1976). 7-Methyl-guanosine and efficiency of RNA translation. *J. Virol.* **19**, 637–642.
- SHIMOTOHNO, K., KODAMA, Y., HASHIMOTO, J., and MIURA, K.-I. (1977). Importance of 5'-terminal blocking structure to stabilize mRNA in eukaryotic protein synthesis. *Proc. Natl. Acad. Sci. USA* **74**, 2734–2738.
- SIT, T. L., and ABOUHAIDAR, M. G. (1993). Infectious RNA transcripts derived from cloned cDNA of papaya mosaic virus: Effect of mutations to the capsid and polymerase proteins. *J. Gen. Virol.* **74**, 1133–1140.
- SKOTNICKI, M. L., MACKENZIE, A. M., and GIBBS, A. J. (1992). Turnip yellow mosaic virus variants produced from DNA clones encoding their genomes. *Arch. Virol.* **127**, 25–35.
- SKOTNICKI, M. L., DING, S.-W., MACKENZIE, A. M., and GIBBS, A. J. (1993). Infectious eggplant mosaic tymovirus and ononis yellow mosaic tymovirus from cloned cDNA. *Arch. Virol.* **131**, 47–60.
- SUMIYOSHI, H., HOKE, C. H., and TRENT, D. W. (1992). Infectious Japanese encephalitis virus RNA can be synthesized from *in vitro*-ligated cDNA templates. *J. Virol.* **66**, 5425–5431.
- SUZUKI, M., KUWATA, S., KATAOKA, J., MASUTA, C., NITTA, N., and TAKANAMI, Y. (1991). Functional analysis of deletion mutants of cucumber mosaic virus RNA3 using an *in vitro* transcription system. *Virology* **183**, 106–113.
- TANGY, F., MCALLISTER, A., and BRAHIC, M. (1989). Molecular cloning of the complete genome of strain GDVII of Theiler's virus and production of infectious transcripts. *J. Virol.* **63**, 1101–1106.
- TANIGUCHI, T., PALMIERI, M., and WEISSMANN, C. (1978). Q β DNA-containing hybrid plasmids giving rise to Q β phage formation in the bacterial host. *Nature* **274**, 2293–2298.
- TOBIN, G. J., YOUNG, D. C., and FLANEGAN, J. B. (1989). Self-catalyzed linkage of poliovirus terminal VPg to poliovirus RNA. *Cell* **59**, 511–519.
- VAN BOKHOVEN, H., VERVER, J., WELLINK, J., and VAN KAMMEN, A. (1993). Protoplasts transiently expressing the 200K coding sequence of cowpea mosaic virus B-RNA support replication of M-RNA. *J. Gen. Virol.* **74**, 2233–2241.
- VAN DER WERF, S., BRADLEY, J., WIMMER, E., STUDIER, F. W., and DUNN, J. J. (1986). Synthesis of infectious poliovirus RNA by purified T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA* **83**, 2330–2334.
- VEIDT, I., BOUZOUBA, S. E., LEISER, R. M., ZIEGLER-GRAFF, V., GUILLEY, H., RICHARDS, K., and JONARD, G. (1992). Synthesis of full-length transcripts of beet western yellows virus RNA: Messenger properties and biological activity in protoplasts. *Virology* **186**, 192–200.
- VERVER, J., GOLDBACH, R., GARCIA, J. A., and VOS, P. (1987). *In vitro* expression of a full-length DNA copy of cowpea mosaic virus B RNA: Identification of the B RNA encoded 24-kd protein as a viral protease. *EMBO J.* **6**, 549–554.
- VIRY, M., SERGHINI, M. A., HANS, F., RITZENTHALER, C., PINCK, M., and PINCK, L. (1993). Biologically active transcripts from cloned cDNA of genomic grapevine fanleaf nepovirus RNAs. *J. Gen. Virol.* **74**, 169–174.
- VOS, P., JAEGLE, M., WELLINK, J., VERVER, J., EGGEN, R., VAN KAMMEN,

- A., and GOLDBACH, R. (1988). Infectious RNA transcripts derived from full-length DNA copies of the genomic RNAs of cowpea mosaic virus. *Virology* **165**, 33-41.
- WEBER, H., HAECKEL, P., and PFITZNER, A. J. P. (1992). A cDNA clone of tomato mosaic virus is infectious in plants. *J. Virol.* **66**, 3909-3912.
- WEILAND, J. J., and DREHER, T. W. (1989). Infectious TYMV RNA from cloned cDNA: Effects *in vitro* and *in vivo* of point substitutions in the initiation codons of two extensively overlapping ORFs. *Nucleic Acids Res.* **17**, 4675-4687.
- WENGLER, G., WENGLER, G., and GROSS, H. J. (1982). Terminal sequences of Sindbis virus-specific nucleic acids: Identity in molecules synthesized in vertebrate and insect cells and characteristic properties of the replicative form RNA. *Virology* **123**, 273-283.
- XIONG, Z., and LOMMEL, S. A. (1991). Red clover necrotic mosaic virus infectious transcripts synthesized *in vitro*. *Virology* **182**, 388-392.
- YAMAYA, Y., YOSHIOKA, M., MESHI, T., OKADA, Y., and OHNO, T. (1988). EXPRESSION of tobacco mosaic virus RNA in transgenic plants. *Mol. Gen. Genet.* **211**, 520-525.
- YOUNG, M. J., KELLY, L., LARKIN, P. J., WATERHOUSE, P. M., and GERLACH, W. L. (1991). Infectious *in vitro* transcripts from a cloned cDNA of barley yellow dwarf virus. *Virology* **180**, 372-379.
- ZIBERT, A., MAASS, G., STREBEL, K., FALK, M. M., and BECK, E. (1990). Infectious foot-and-mouth disease virus derived from a cloned full-length cDNA. *J. Virol.* **64**, 2467-2473.
- ZIEGLER-GRAFF, V., BOUZOUBAA, S., JUPIN, I., GUILLEY, H., JONARD, G., and RICHARDS, K. (1988). Biologically active transcripts of beet necrotic yellow vein virus RNA-3 and RNA-4. *J. Gen. Virol.* **69**, 2347-2357.