***In vitro* expression of cauliflower mosaic virus genes**

K.Gordon^{1,2}, P.Pfeiffer¹, J.Fütterer² and T.Hohn²

¹Laboratoire de Virologie, Institute de Biologie Moléculaire et Cellulaire du CNRS, 15 rue Descartes, 67084 Strasbourg, France and

²Friedrich Miescher-Institut, PO Box 2543, 4002 Basel, Switzerland

Communicated by T.Hohn

The eight major open reading frames (ORFs) of cauliflower mosaic virus (CaMV) have been cloned for *in vitro* transcription and translation. All the ORFs could be translated. Using antisera against either purified virus or specific gene products, the translation products were screened by immunoprecipitation. The products of ORFs III, IV and V were confirmed as components of the virions. Molecular weights of primary translation products were determined and compared with those found *in vivo*. A further series of constructs was designed to test whether translation of adjacent ORFs is coupled in a relay-race fashion as proposed on the basis of earlier *in vivo* mutagenesis studies. Downstream genes on dicistronic RNAs could be translated, although inefficiently. In view of the similarity between the arrangement of the CaMV coat protein and reverse transcriptase genes and the corresponding genes of retroviruses, we asked whether the CaMV reverse transcriptase could be expressed *in vitro* as a fusion protein, e.g. by ribosomal frame shifting. No such fusion was observed, suggesting that the polymerase gene is translated from its own ATG. Key words: polycistronic mRNA/translational control/reverse transcriptase/RNase H/retrovirus

Introduction

Mature cauliflower mosaic virus (CaMV) virions contain a double stranded DNA genome of ~8000 bp, which is replicated by reverse transcription of an 8200 nt (35S) RNA containing a 180 nt terminal redundancy. There are many similarities between CaMV and other retroid elements with respect to their mode of replication and to the organization of part of their coding sequences (Mason *et al.*, 1987; Bonneville *et al.*, 1987; Fütterer and Hohn, 1987). While the mechanism of CaMV replication via reverse transcription is partly understood (Hohn *et al.*, 1985; Bonneville *et al.*, 1987), virtually nothing is known about the expression mechanism of viral proteins. The CaMV genome has the capacity to encode, depending on the strain, 7–8 proteins of more than 10 kd (Figure 1). In infected plants protein products from open reading frames (ORFs) I–VI have been detected (Table II).

Mutagenesis studies have shown that expression of the first ORFs (VII to at least III) is coupled in a relay-race fashion: mutants in the dispensable ORFs VII and II were viable only if translation of each of these ORFs terminated before the

start of the following, essential gene (ORFs I and III, respectively) (Sieg and Gronenborn, 1982; Dixon and Hohn, 1984). Furthermore, there was found to be a limit on the size of intergenic regions which could be tolerated (Brisson *et al.*, 1984). These studies led to the proposal that these CaMV ORFs are expressed one after the other from a polycistronic mRNA. ORFs III and IV and ORFs IV and V overlap slightly in the wild-type virus; it may therefore be necessary to modify the relay-race model to explain their translation.

The most likely candidate for a polycistronic mRNA is the only major transcript covering ORFs VII–V, the 35S RNA. CaMV would therefore further resemble the retroviruses, whose genomic RNAs also are mRNAs for the *gag* and *pol* proteins. Furthermore, the product of ORF VI is translated from an abundant subgenomic mRNA (19S), reminiscent of the translation of retroviral *env* protein from a separated, spliced mRNA. Besides the genomic 35S and the 19S RNA some virus-specific minor RNAs have been described (Condit *et al.*, 1983; Plant *et al.*, 1985). It is, however, unclear whether these could act as mRNAs for the other viral proteins.

In vitro translation of mRNAs from infected plants yields ORF VI protein as the only virus-specific product. No product of any of the other viral genes has been detected upon *in vitro* translation of *in vivo* RNA. It was not known whether this was due to the viral ORFs being untranslatable, to inhibition of translation by the 600-nt long leader sequence of the 35S RNA, or to problems arising from the polycistronic arrangement of genes on this RNA.

Since no conditional CaMV mutants have been developed, and no cell culture or protoplast system suitable for gene expression studies was available at the time, we chose an *in vitro* approach using SP6 or T7 RNA polymerase to produce RNAs covering specific segments of the viral genome. These *in vitro* transcripts were then translated *in vitro* using the reticulocyte lysate or wheat germ extract, in order to determine whether each ORF could be translated and whether downstream ORFs on dicistronic mRNAs could be translated as efficiently as the upstream ones. Using this approach, we were able to establish that proteins could be translated *in vitro* from all major ORFs (I–VIII) of CaMV, including those (VII and VIII) whose products have not yet been detected *in vivo*. Immunoprecipitation with an antiserum against the virus was used to show that some of these proteins are associated with the virus *in vivo*. These translation products are also proving useful in studying the expression and processing of viral gene products in plants. This is especially the case for the extensively processed products of ORF IV, whose full-length product has now been shown to migrate more slowly on SDS–polyacrylamide gels than previously thought, and for the products from ORF V, which gives rise to a number of different protein species in plants (Pietrzak and Hohn, 1987). Translation *in vitro* of dicistronic transcripts, however, showed that the downstream genes

were translated with low efficiency, suggesting that these translation systems are not suitable for studying reinitiation. The possibility that translation of the overlapping ORFs IV and V could yield a fusion protein by a +1 frameshift, analogous to the -1 shift observed for retroviral *gag-pol* genes (Jacks and Varmus, 1985; Jacks *et al.*, 1987) and to the +1 shift observed for the major reading frames (A and B) of yeast Ty elements (Clare and Farabaugh, 1985; Mellor *et al.*, 1985), was studied in some detail. No such frameshift was observed for the CaMV genes.

CaMV genome segments covering each of the eight major ORFs were cloned downstream from the bacteriophage SP6 or T7 promoters to give a series of constructs (Figure 1, Table I), which could be transcribed *in vitro* to yield specific mRNAs for each ORF. The transcripts from the eight plasmids had different 5' untranslated leaders, derived from the CaMV sequences immediately upstream of the ORF under study and varying in length from 10 to 155 nt. In all cases, however, the leaders lacked any ATG codons upstream of that initiating the first ORF. These transcripts were designed with the aim of obtaining sufficient translation products for analysis, rather than of comparing their relative efficiencies of translation. The constructs were stable in *Escherichia*

In the cases of ORFs I, IV, V and VI substantial amounts of smaller than full length translation products were obtained in addition to the full size one. This effect was mainly seen in the reticulocyte lysate but was also apparent in the wheat germ system. These smaller species could have been derived from translation initiation at internal ATGs (possibly on degraded RNAs), from premature translation termination or from protein processing. Time course studies on the translation of ORF I in the reticulocyte lysate (data not shown) revealed that the truncated products arose with the same kinetics as the full-length one, suggesting that they were not processing products. On the other hand, 3'-terminal truncation of the ORF I transcript by 26 or 174 nt led to a corresponding reduction in size of the shorter translation products, showing that they were carboxy-coterminal and therefore probably derived from initiation at internal ATGs.

Table I. Plasmids used to produce mRNAs for CaMV ORFs

Plasmid	ORF studied	CaMV restriction fragment	Vector (site) used	Site ^f opened	RNA length	Polymerase
pTCa7	VII	<i>Nla</i> III	pGEM2(<i>Hind</i> III – <i>Sac</i> I) ^g	(<i>Sac</i> I)	326	T7
pTCa7.1	VII,I	<i>Nla</i> III – <i>Xho</i> I ^c	pGEM2(<i>Hind</i> III – <i>Sac</i> I) ^g	(<i>Bam</i> HI)	1675	T7
pSCa1	I	<i>Bam</i> HI	pSP65(<i>Bam</i> HI) ^h	(<i>Sac</i> I)	1190	SP6
pSCa286/1	VII ^a ,I	<i>Cla</i> I – <i>Hinc</i> II ^c	pSP64(<i>Hind</i> III – <i>Sac</i> I) ⁱ	(<i>Pvu</i> II)	1402	SP6
pSCa373/1	VII ^a ,I	<i>Cla</i> I – <i>Hinc</i> III ^c	pSP64(<i>Hind</i> III – <i>Sac</i> I) ⁱ	(<i>Pvu</i> II)	1392	SP6
pTCa1.2	I,II	<i>Sac</i> I – <i>Hpa</i> I	pGEM2(<i>Sma</i> I)	<i>Stu</i> I	2238	T7
pSCa2.3	II,III	<i>Bgl</i> II – <i>Eco</i> RI	pSP64(<i>Bam</i> HI – <i>Eco</i> RI)	<i>Eco</i> RI	1150	SP6
pSCa3	III	<i>Xba</i> I – <i>Eco</i> RI	pSP64(<i>Xba</i> I – <i>Eco</i> RI)	<i>Eco</i> RI	747	SP6
pTCa3.4	III,IV ^b	<i>Cla</i> I – <i>Hpa</i> II	pT7-2(<i>Acc</i> I)	(<i>Sac</i> I)	1755	T7
pmic4.5	IV,V	<i>Bam</i> HI – <i>Sac</i> I	pmic/pSP64 ^j	<i>Eco</i> RV	3599	SP6
pSCa4Δ.5	IV ^c ,V	<i>Hind</i> III – <i>Sac</i> I ^c	pGEM1(<i>Hind</i> III – <i>Sac</i> I)	<i>Eco</i> RV	3550	SP6
pSCa(6)/4.5	IV,V	<i>Xba</i> I – <i>Eco</i> RV ^c	pSP64/65 ^k	(<i>Pvu</i> I)	4820	SP6
pSCa5	V	<i>Xba</i> I – <i>Sac</i> I ^c	pSP64(<i>Xba</i> I – <i>Sac</i> I)	<i>Eco</i> RV	2155	SP6
pSCa5b	V	<i>Sma</i> I – <i>Sac</i> I	pSP64(<i>Sma</i> I – <i>Sac</i> I)	<i>Eco</i> RV	2121	SP6
pSCa6	VI	<i>Eco</i> RV	pSP65(<i>Sma</i> I)	(<i>Sac</i> I)	1675	SP6
pSCa8.5	VIII,V ^d	<i>Hind</i> III	pSP64(<i>Hind</i> III)	<i>Pvu</i> II	750	SP6

^aORF VII is truncated (see Figure 1).

^bC-Terminus of ORF IV replaced by that of the β -galactosidase gene.

^cDeleted internal *Pst*I fragment.

^dThe reading frame begun in ORF V continues beyond the *Hind*III site into and ends within the vector sequences.

^eThese plasmids were constructed in several steps because of the presence of internal sites for the restriction endonucleases used.

^fSites in brackets are from vector.

^gThe CaMV fragment was first cloned into the *Sph*I site of pUC19 and then excised as *Hind*III/*Sac*I fragment for cloning into the corresponding sites of pGEM2.

^hDescribed in Martinez *et al.* (1987).

ⁱThese constructs contain a 116 bp *Eco*RV – *Sac*I fragment from pSCa5 linking the *Hinc*II site of the insert to the *Sac*I site of the vector.

^jDetails will be published separately (Fütterer, in preparation).

^kThe CaMV *Eco*RV(5712)/*Bgl*II(4495) restriction fragment was cloned into the *Hinc*II/*Bam*HI sites of pSP64 and the CaMV *Xba*I(3586)/*Eco*RV(5712) restriction fragment into the *Sma*I site of pSP65; the two fragments were then joined via the *Sma*I/*Xba*I polylinker of pUC19.

The availability of antibodies specific for the carboxy- and amino-termini of ORF V (Ziegler *et al.*, 1985; Pietrzak and Hohn, 1987) allowed us to study the smaller products derived from this ORF in more detail. Both antibodies reacted with the 80-kd band, confirming the veracity of the ORF V construct. The antiserum against the amino-terminus reacted specifically with truncated polypeptides of 70, 22 and 20 kd, whereas that against the carboxy-terminus reacted specifically with another polypeptide of 58 kd (Figure 3B), which is produced in significant amounts in the reticulocyte lysate by transcripts from both pSCa5 and pSCa5b. The polypeptides of 70, 22 and 20 kd are therefore derived from the amino-terminus of ORF V, whereas the 58-kd peptide is derived from the carboxy-terminus. The 58-kd and 22-kd proteins may therefore be produced by a single cleavage of the full-length ORF V product, although initiation at an internal ATG (the 58-kd protein) and premature termination (the 22-kd protein) remain formally possible. Further experiments will be needed to resolve this problem. Premature termination of translation could also be invoked to explain the 20-kd and 70-kd proteins. The latter is present in only very low amounts compared to the other translation products (Figure 3).

Identification of virus components

Translation products from all ORFs were tested with an antiserum directed against dissociated virus particles. The gene products of ORFs III, IV and V were specifically and efficiently precipitated by this antiserum (Figure 4), suggesting that these gene products are components of the virus. In con-

trast, the products of ORFs I, II, VI, VII (Figure 4) and VIII (not shown) were not precipitated, suggesting that they are not components of the virions. The antiserum AV in Figure 4 was the only one of the six tested which immunoprecipitated the ORF VI product (albeit only to a very minor extent), showing that the virus used in its preparation was slightly contaminated with viroplasm protein which is produced in very large amounts in CaMV-infected tissue, yet it did not recognize any of the gene products from ORFs I, II, VII or VIII. In control experiments, preimmune serum was found not to immunoprecipitate any of the viral gene products.

Expression of tandemly arranged CaMV open reading frames

As mentioned in the Introduction, CaMV ORFs with the exception of ORF VI are thought to be translated from the genomic polycistronic RNA. On this RNA the individual ORFs are either separated by a short intergenic region or overlap slightly. Where there is a short intergenic region, the distance between the termination codon of the upstream ORF and the first ATG of the downstream ORF varies from 1 nt (ORFs I/II, II/III) to 60 nt (ORFs VII/I). In contrast, the first ATG of ORF IV is 6 codons upstream of the termination codon of ORF III, and the first ATG of ORF V is 12 codons upstream of the termination codon of ORF IV. ORF IV is in the –1 frame relative to ORF III, whereas ORF V is in the +1 frame relative to ORF IV. To test whether the heterologous *in vitro* translation systems can respond sufficiently to these varied structural features of the

Fig. 2. Translation products of CaMV ORFs (roman numbers). Aliquots (1 μ g) of the transcripts from CaMV ORFs I–VIII were translated in 10 μ l of the wheat germ extract containing 10 μ Ci [35 S]methionine. Half of the total translation mix was analysed on 12.5% SDS–PAGE. The transcripts used were derived from the following plasmids: VII: pTCa7; I: pSCa1; II: pSCa2.3 [linearized at the *Bam*HI site (1927) in ORF III]; III: pSCa3; IV: pmic4.5 [linearized at the *Pvu*II site (3993) in ORF V]; V: pSCa5; VIII: pSCa8.5'; VI: pSCa6.

Table II. Molecular weights of the CaMV gene products

ORF	Mol. wt calculated	Mol. wt (kd) found		Reference ^b
		<i>in vitro</i>	<i>in vivo</i> ^a	
I	36.7	41	<u>41–46</u> /42/36–38	1–3
II	17.8	19	18	4
III	14.1	14	<u>15</u> /11 ^c	5
IV	56.7	80	58/ <u>44</u> /37 ^d	6–9
V	78.6	79	80/62/ <u>58</u> /22/18	10–13
VI	58	62	62–66	1, 14–17
VII	11.4	14		
VIII	12.5	16		

^aThe most abundant forms of each protein are underlined.

^bReferences: 1. Harker *et al.*, 1987; 2. Young *et al.*, 1987; 3. Martinez-Izquierdo *et al.*, 1987; 4. Givord *et al.*, 1984; 5. Giband *et al.*, 1986; 6. Al Ani *et al.*, 1979; 7. Hahn and Shepherd, 1982; 8. Menissier-de Murcia *et al.*, 1986; 9. Martinez-Izquierdo and Hohn, 1987; 10. Menissier *et al.*, 1984; 11. Pfeiffer *et al.*, 1984; 12. Ziegler *et al.*, 1985; 13. Pietrzak and Hohn, 1987; 14. Al Ani *et al.*, 1980; 15. Odell and Howell, 1980; 16. Covey and Hull 1981; 17. Xiong *et al.*, 1982.

^cThe 15 kd protein is most abundant in the viroplasm, whereas that of 11 kd is associated with the virions.

^dAn 80 kd protein species detected *in vivo* has been interpreted as a dimer of the processed 37 kd species (Al Ani *et al.*, 1979). This would mask the full-length gene product which we have observed *in vitro*.

RNA to allow efficient translation of the downstream ORFs, a series of plasmids containing pairs of adjacent CaMV ORFs (Figure 1, Table I) was tested. Because of the instability of ORF IV constructs and because the primary translation products of ORFs, IV and V comigrate, it was necessary to modify ORF IV. Its carboxy-terminal portion was replaced by the bacterial β -galactosidase gene in pTCa3.4', its amino-

Fig. 3. (A) Translation products from the two ATGs at the start of ORF V. Transcripts from pSCa5 and pSCa5b (indicated as 5 and 5b). (B) Immunoprecipitation of translation products from pSCa5 with different antisera. TM: 1 μ l of translation mix; immunoprecipitations of 10 μ l aliquots of translation mix were performed using the following antisera (see Materials and methods for details): **Anti-Virus**: antiviral antiserum; **Anti-V_{NT}**: antiserum against the amino-terminus of ORF V; **Anti-V_{CT}**: antiserum against the carboxy-terminus of ORF V; **PI**: preimmune antiserum [from the rabbit which was used to raise Anti-V_{NT}; a separate preimmune antiserum for **Anti-V_{CT}** was also negative (not shown), as was that for antiviral antiserum, see Figure 4]. Translation was in the reticulocyte lysate system. Electrophoresis was on 10% SDS–PAGE. The 80- and 78-kd (full length) and 70-, 58-, 22- and 20-kd (sublength) products are marked.

terminal portion by CaMV ORF VI in pSCa(6)/4.5 and its most basic portion was deleted in pSCa4 Δ .5. Plasmid pmic4.5 contains antisense sequences to the 5' terminus of this ORF under constitutive control.

RNA from these constructs directed synthesis of normal amounts of protein from the respective upstream ORFs and low amounts of the downstream ones (Table III). The only pair of ORFs for which higher levels of translation were generally observed was the VII/I combination. In view of the previously suggested initiation at internal ATGs in the monocistronic cases (see above) we asked what proportion of translation of the downstream ORF was due to reinitiation by ribosomes which had completed translation of the upstream ORF.

This question was addressed by comparing translation of equal molar amounts of [3 H]RNA derived from constructs based on deletion and linker insertion mutants that had been developed and analysed *in vivo* (Dixon and Hohn, 1984). In the test case, the upstream ORF VII overlapped with the start of ORF I by 106 nt (pSCa286/a, derived from the non-viable mutant pCa286); in the control (pSCa373/1, derived from the viable mutant pCa373), ORF VII terminated before the start of ORF I (as in the natural situation, although with a shorter intergenic region). Equal molar amounts of the [3 H]-labelled RNA were translated *in vitro* and the amounts of [3 S]-labelled translation product quantified as described in the Materials and methods. In pSCa286/1 where ORF VII overlaps with ORF I, translation of the downstream ORF I was reduced by 60% and 40% (compared to the control, pSCa373/1) in the wheat germ and the reticulocyte lysate systems, respectively (Table III). This suggests that only about half of the downstream (ORF I) translation product in the natural case was in fact derived from relay-race translation and the other half from internal initiation, e.g. by

Fig. 4. Immunoprecipitation of CaMV translation products by antivirus antiserum. Transcripts of the CaMV ORFs were translated in the wheat germ system and the products immunoprecipitated prior to analysis by SDS-PAGE (see Materials and methods for details). Blocks for the products derived from constructs containing the individual ORFs are marked with the roman ORF number. Blocks represent individual experiments. The full length translation products are marked with arrowheads; see Figure 2 for a comparison of the mobilities of the full length proteins. Tracks show: T: 1 µl of the individual translation mix without further treatment; P: 10 µl of the same immunoprecipitated with preimmune antiserum from the rabbit used to raise the antivirus antiserum; AV: 10 µl of the same immunoprecipitated with antiserum against purified virions.

ribosomes which leaked past the first (ORF VII) ATG on the RNA.

An alternative possibility that must be considered for translation of overlapping reading frames is the production of a fusion protein by ribosomal frame shifting before the termination codon of the upstream ORF. Dicistronic constructs covering ORFs IV and V contained modified forms of ORF IV for the above mentioned reasons. In pSCa(6)/4.5 the initiation codon of ORF VI (plus a few amino acids) was fused in-frame to the carboxy-terminal 25 codons of ORF IV. *In vitro* transcription yielded a RNA encoding two proteins, the ORF VI::IV fusion protein and the ORF V protein. Both *in vitro* translation systems produced the ORF VI::IV fusion protein very efficiently from this RNA, but only the reticulocyte lysate yielded a little of the ORF V product starting from the first ORF V AUG (Figure 5). Neither system produced ORF VI::IV::V fusion proteins, nor translation products starting at the second ATG of ORF V, showing that initiation or reinitiation of ORF V, where it happened, was upstream from the ORF IV stop codon. Translation of pSCa4Δ.5 (which has the most basic portion of ORF IV deleted) in the reticulocyte lysate yielded surprisingly high levels of the ORF V product (Figure 4, Table III). Immunoprecipitation of translation products from pmic4.5 with the antiserum against the amino-terminus of ORF V showed that some ORF V product was present. No evidence was found in these cases for any ORF IV::V fusion protein analogous to the *gag/pol* polyprotein of retroviruses (Figure 5), even though a control translation of RNA from pGP, which includes the RSV *gag/pol* junction (Jacks and Varmus, 1985) yielded the expected fusion protein in the ratio reported (not shown). Moreover, time course studies (not shown) did not disclose any fusion protein at early stages of translation.

A possible alternative strategy for ORF V expression involves the short ORF VIII located immediately upstream of ORF V in the same reading frame, and terminated by a potentially suppressible TAG codon. The construct pSCa8.5' that covers ORF VIII and the first 170 codons of ORF V yielded a translation product from ORF VIII but no clear evidence was detected for any ORF VIII::V fusion protein (Figure 5).

Table III. Translation of downstream genes on dicistronic transcripts

Plasmid	Expression of downstream/upstream ORF (%)	
	Wheat germ	Reticulate lysate
PTCa7.1	10	20 ^a
pTCa1.2	5	n.d.
pSCa2.3	10	n.d. ^b
pTCa3.4	5	n.d. ^b
pSCa(6)/4.5	< 1	5
pSCa4Δ.5	5	20
pSCa8.5' ^c	10	n.d. ^b
pSCa373/1 ^d	4	4
pSCa286/1 ^d	1.5	2.5

n.d. not determined.

^aSince the translation product of ORF VII could not be detected in the reticulocyte lysate, it was necessary to compare translation of equal molar amounts of an RNA showing the natural arrangement of ORFs VII and I and another in which ORF VII was fused in phase to ORF I, which could then act as a reporter gene for translation initiating at the ORF VII ATG.

^bSince the band corresponding to the upstream ORF could not be detected, the relative expression of the downstream ORF could not be quantified, although inspection of the gel showed it to be very low.

^cTranslation of only the amino-terminal fragment of ORF V as present on this construct was analysed.

^dThe truncated ORF VII in these constructs was translated more efficiently than the full-length ORF VII in pTCa7.1, without any significant increase in ORF I translation.

Test for enzymic activity of proteins produced *in vitro*

To detect whether any DNA polymerase or RNase H activity was associated with the translation products of ORF V, these were analysed on an activity gel in parallel with various types of nuclear extracts obtained from healthy (H) or infected (V) plants. In this assay, DNA polymerases are renatured *in situ* after SDS gel electrophoresis and incorporate ³²P-labelled dNTPs into a TCA-insoluble template/product complex. The dry gel was autoradiographed using two superimposed films. The ³⁵S label in the translation products was detected only on the film adjacent

Fig. 5. Translation of RNAs covering ORFs IV and V. *In vitro* transcripts from pmic4.5, pSCa4Δ.5, pSCa8.5' and pSCa(6)/4.5 were translated in either the wheat germ (W) or reticulocyte lysate (R) systems and the products analysed by electrophoresis on 12% polyacrylamide gels. The positions of the full length ORF IV, V and VIII products (pIV, pV, pVIII; pIV and pV comigrate), as well as those of the internally deleted ORF IV product (pIV'), the amino-terminal fragment from ORF V (pV') and the ORF VI–IV fusion (pVI–IV), are shown. E is an endogenous band present in the reticulocyte lysate translation mixtures.

to the gel, whereas the ^{32}P label was detected on both. Various extracts from CaMV-infected plants, run as markers, showed DNA polymerase activity. The CaMV-induced reverse transcriptase present in extracts from infected tissue and in purified virions has previously been shown to comigrate on activity gels with the Klenow fragment and host DNA polymerases at about 75 kd (Menissier *et al.*, 1984; Pfeiffer *et al.*, 1984; Hohn *et al.*, 1985). Neither of the translation products from ORF V showed any DNA polymerase activity (Figure 6). Western blotting (not shown) of other gels on which the translation products and plant extracts were electrophoresed confirmed that *in vitro* translation yielded protein derived from ORF V in equivalent amounts to those present in the plant extracts.

The radioactive band that reveals the position of the reverse transcriptase activity in the extracts from virus-infected plants had an apparent mol. wt of 75 kd, i.e. slightly smaller than that of the *in vitro* translation products derived from ORF V. This indicates that the primary translation product from ORF V undergoes *in vivo* modification, which might be crucial for the enzymic activity.

Analysis of RNase H activities (Figure 7) showed that the endogenous activity present in uninfected plants (track d) was significantly stimulated by infection not only with CaMV (track e) but also with unrelated RNA viruses (not shown). Interestingly, there did not appear to be any activity with a mobility comparable to that of the reverse transcriptase at 75–80 kd. The *in vitro* translation products from ORF V did not show any RNase H activity (tracks a and b).

Discussion

All CaMV ORFs larger than 90 codons could be translated *in vitro* from suitable RNAs. This includes the small ORFs

Fig. 6. Analysis of translation products from ORF V for potential DNA polymerase activity. Transcripts from pSCa5 (5.1) or pSCa5b (5.2) were translated in the wheat germ system in the presence of [^{35}S]methionine. The translation products were then submitted to DNA polymerase activity gel analysis (see Materials and methods) in parallel with plant extracts (N.E.) prepared by hypotonic leaching of organelle suspensions of healthy (H) and CaMV-infected (V) turnip leaves. C.L.: cleared lysate prepared by salt lysis of these organelles; Spd: as N.E. but leached in the presence of 2 mM spermidine; DOC: as N.E. but leached in the presence of deoxycholate. Pol: *E. coli* DNA polymerase, Kle: Klenow fragment of the *E. coli* DNA polymerase. After SDS–PAGE, the proteins were renatured and DNA synthesis allowed to proceed. The gel was then washed and dried, before autoradiography using two superimposed films. The upper half (^{35}S + ^{32}P) shows the film immediately adjacent to the gel (detecting both labels), whereas the lower half (^{32}P) shows the second film, detecting only this label.

VII and VIII, which are dispensable for virus infectivity and have not yet been found in infected plants. The *in vitro* translation systems usually do not provide for the protein maturation and processing events that occur *in vivo*. Thus the original full length translation products of individual ORFs become apparent and can be compared with the proteins accumulating *in vivo*. Translation products from ORFs I, II, III and VI were found to have similar mobilities to the corresponding proteins isolated from infected plants although for ORF I and III shorter minor components have also been observed (Harker *et al.*, 1987; Giband *et al.*, 1986).

Comparison of the *in vitro* and *in vivo* products from ORFs IV and V, on the other hand, indicates that extensive protein processing of these gene products occurs *in vivo*, yielding material shorter than the primary translation products, which both migrate at 80 kd on SDS–polyacrylamide gels. In the case of ORF IV, the previously described pp58 cannot be the primary translation product, as was assumed (Hahn and Shepherd, 1982; Martinez-Izquierdo and Hohn, 1987) but already a processing product. Translation of ORF V in the reticulocyte lysate yielded a number of smaller proteins in addition to the full length product. Intriguingly, two of these,

Fig. 7. Analysis of the translation products of ORF V for potential RNase H activity. The ^{35}S -labelled translation products from pSCa5 (track a) or pSCa5b (track b) were analysed by activity gel analysis on a 10% polyacrylamide gel containing 4 $\mu\text{g/ml}$ of [^{32}P]RNA—unlabelled DNA hybrid (see Materials and methods for details). Track c contains the wheat germ extract alone. The other tracks contain extracts from uninfected plants (track d) or from CaMV-infected plants (track e). Proteins derived from pSCa5 (5.1) and pSCa5b (5.2) can be seen as dark bands in tracks a and b, respectively.

the amino-terminal 22-kd product and that of 58 kd from the carboxy-terminus, corresponded in size and position to some of the products derived from ORF V observed *in vivo* (Ziegler *et al.*, 1985; Pietrzak and Hohn, 1987). Experiments to determine whether these *in vitro* and *in vivo* products arise by similar processing pathways from the primary translation product are in progress. Since the 22-kd protein covers the region of ORF V which has been found to show homology to retroviral proteases, it may be the liberated, active form of the protease (Toh *et al.*, 1985; Hull and Covey, 1986; Fütterer and Hohn, 1987).

The abnormally slow mobility of several of the CaMV translation products, especially for proteins translated from ORF IV, may be due to the uneven distribution of acidic and basic amino acid residues. In the case of ORF IV, the very acidic amino-terminal region of 250 amino acids is followed by a very basic region of 90 amino acids and then an acidic carboxy-terminus (Franck *et al.*, 1980). The anomalous mobility means that the shorter forms found in virions may be even more extensively processed than at first thought (Al Ani *et al.*, 1979; Martinez-Izquierdo and Hohn, 1987). The amino-terminal sequence of the 44-kd derivative from ORF IV has been determined (Martinez-Izquierdo and Hohn, 1987) but it will be necessary to sequence both termini of this and other proteins from this ORF to determine their actual location. The product of the analogous gene on the yeast transposable element Ty1-15, TyA [expected mol. wt 50.4 kd (Mellor *et al.*, 1985)] also migrates anomalously slowly at 62 kd (Müller *et al.*, 1987). The product of this gene also has an uneven distribution of acidic and basic amino acids. Similar observations have been made for the product of RNA 2 of cucumber mosaic virus (Rezaian *et al.*, 1984) and brome mosaic virus (Ahlquist *et al.*, 1984), and for that of the yeast *GCN4* gene (Hope and Struhl, 1985).

Immunoprecipitation studies using antiserum against highly purified, SDS-dissociated virus particles showed that the products of ORFs III, IV and V are components of the virion.

This confirms studies on the individual ORFs III (Giband *et al.*, 1986), IV (Al Ani *et al.*, 1979) and V (Menissier *et al.*, 1984). The other gene products were not found to be associated with the virus. Proteins derived from ORF I (Harker *et al.*, 1987; Martinez-Izquierdo *et al.*, 1987; Young *et al.*, 1987), ORF II (Givord *et al.*, 1984) and ORF VI (Xiong *et al.*, 1982) have been shown to be associated with inclusion bodies and only loosely, if at all, attached to virus particles, although Young *et al.* (1987) claimed that a processed form of the ORF I product is a component of the virus particle. Antiserum against the virion did not immunoprecipitate the ORF I translation product at all, even though the virion preparation was still slightly contaminated with viroplasm protein and the antiserum thus weakly recognized the ORF VI product: we therefore conclude that the ORF I product is not a component of the virion.

Translation of some transcripts in both the wheat germ and the reticulocyte lysate systems yielded significant amounts of products arising from initiation of translation at internal ATGs, although the effect was more marked with the reticulocyte system. This is consistent with the observation of Lütcke *et al.* (1987), that the reticulocyte lysate is more discriminatory in recognizing the context of an initiation codon and thus more likely to ignore an unsuitable upstream codon than the wheat germ system. It is possible that leakage of ribosomes past upstream ATG codons to initiate internally may be significant *in vivo* for CaMV, but this cannot be reconciled with the results of mutagenesis carried out by Sieg and Gronenborn (1982) and Dixon and Hohn (1984).

Downstream genes on dicistronic RNAs have been found to be translated rather poorly *in vitro*, although we observed that some more significant translation of ORF I downstream from ORF VII and of ORF V downstream from ORF IV occurs. However, at this stage we do not believe that the translation systems used properly reflect the situation in CaMV-infected cells. *In vivo* downstream translation might be enhanced drastically by host- and virus-coded factors lacking from the *in vitro* translation systems. Providing these hypothetical factors in the *in vitro* systems could become a powerful tool in understanding the mechanisms of CaMV translational control. Their lack might also be the major reason why CaMV 35S RNA and total CaMV mRNA (with the exception of 19S RNA) is translated so poorly *in vitro* (Franck *et al.*, 1980; our observations). Indeed, on the complete 35S RNA, the largest ORFs (IV and V), whose products would be those most easily identified against a background of host translation products, are also the most posterior (apart from ORF VI, which is expressed via a distinct mRNA). Other studies, to be published elsewhere, have shown that the long 5' untranslated sequence on the 35S RNA also reduces translation of downstream genes *in vitro*.

Transient expression studies in plant protoplasts (French *et al.*, 1986) have shown significant levels (65%) of translation of a reading frame second in position after a short upstream open reading frame in comparison to the level in absence of the small ORF (100%). In animal cells, however, lower levels of reinitiation have been observed, i.e. 10–20% (Peabody and Berg, 1986).

The *gag* and *pol* genes of avian retroviruses correspond to the CaMV ORFs IV and V and overlap to a similar extent. While the avian retrovirus *pol* gene product is produced as a *gag-pol* fusion protein *in vitro* and *in vivo*, we have

not been able to detect any fusion protein from ORFs IV and V. This suggests that CaMV may express ORF V starting from its own ATG, rather than as part of a fusion protein with ORF IV. Although it remains possible that such an *in vitro* fusion protein is rapidly or even cotranslationally processed, the slow apparent processing of ORF V to yield the 58-kd and 22-kd proteins (our unpublished observations; see also Figure 3, which shows that a large amount of full length ORF V product remains visible after 90 min of translation) would argue against this. There may still be more surprises concerning ORF V expression and protein maturation: the active form of the reverse transcriptase actually appears slightly smaller than the direct translation product of ORF V, from which it is derived and the *in vitro* ORF V product is not enzymatically active. Further studies will be needed to determine how this protein is activated. Processing of the primary gene product to yield active reverse transcriptase is a general characteristic of retroviruses (Copeland *et al.*, 1985; Tanese *et al.*, 1985; Farmerie *et al.*, 1987).

The *in vitro* translation products have the potential to serve many other purposes, in addition to allowing tests of putative enzymic activity. They could be used as antigens to produce antibodies, or as substrates to study CaMV-specific protein processing and modification and the assembly of inclusion bodies and virus particles.

Materials and methods

Virus

CaMV (Cabb-S) was propagated in turnip plants (*Brassica rapa* cv Just Right) by mechanical inoculation of plantlets at the three-leaf stage. Virus was extracted from frozen leaves 4–5 weeks p.i. by the Triton X-100/urea method of Hull *et al.* (1976) and purified by differential centrifugation. The virus in the high speed pellet was further purified by sucrose gradient centrifugation and buoyant density centrifugation as described by Al Ani *et al.* (1979). The very labile 55-kd (P_6) viral protein (Al Ani *et al.*, 1979) showed up clearly upon SDS–PAGE of dissociated virus, confirming that proteolysis of the virus preparation had been kept to a minimum.

Preparation of antisera

Anti virus. CaMV (5 mg) purified by sucrose gradient and CsCl centrifugation as described above was dissociated by boiling for 5 min in 1% SDS, 1% β -mercaptoethanol, mixed with one volume of Freund's incomplete adjuvant and injected i.m. to rabbits for 5 weeks at 1-week intervals.

anti-ORF V. An antibody raised in rabbits against a synthetic peptide covering the carboxy-terminal 25 amino acids of ORF V (Ziegler *et al.*, 1985) was a kind gift of Drs V. Ziegler and K. Richards. An antibody specific for the amino-terminal 403 amino acids of ORF V was raised in rabbits using an ORF V:: β -galactosidase fusion protein expressed in bacteria (Pietrzak and Hohn, 1987).

For all antisera, preimmune antiserum was collected from the rabbit before starting the course of immunization injections.

Isolation of CaMV DNA

DNA was extracted from the purified virus as described by Hohn *et al.* (1980).

Cloning of CaMV genes for *in vitro* expression

Fragments of CaMV DNA bearing selected ORFs (Figure 1, Table I) were prepared by digestion of CaMV DNA or of DNA from mutants described in Dixon *et al.* (1983) and Dixon and Hohn (1984) with restriction endonucleases (under the conditions described by the suppliers) and purified on low-melting point agarose gels. Such fragments were cloned into the RNA expression vectors pSP64, pSP65 (Melton *et al.*, 1984), pT7-2 (Tabor and Richardson, 1985), pGEM1 and pGEM2 (Promega Biotec), using standard methods (Maniatis *et al.*, 1982). After amplification, the plasmids were purified and linearized with the appropriate restriction endonucleases before *in vitro* transcription in the presence of 500 μ M NTPs using the SP6 or T7 polymerases as described by the manufacturers. In some experiments, capped transcripts were produced by addition of 500 μ M m⁷G(5')ppp(5')G

to the transcription reaction. After analysing the *in vitro* transcripts by agarose gel electrophoresis, they were purified from the transcription mix by phenol/chloroform extraction and recovered by ethanol precipitation.

For quantitative evaluation of the translational efficiency of some mRNAs, transcription was performed in the presence of 200 μ Ci/ml [³H]UTP and 20–500 μ M unlabelled UTP. Unincorporated [³H]UTP was removed from the transcript by several cycles of ethanol precipitation in 2 M ammonium acetate (Maniatis *et al.*, 1982), before scintillation counting of aliquots of the transcript.

In vitro translation

Reticulocyte lysate. Translation reactions in the nuclease-treated reticulocyte lysate (Pelham and Jackson, 1976) contained 60% (v/v) lysate (from Green Hectares, Oregon, WI, USA), 15 μ M hemin, 15 mM Hepes–KOH, pH 7.5, 120 mM potassium acetate, 1 mM magnesium acetate, 2.8 mM creatine phosphate, 30 μ g/ml creatine kinase, 0.8 mM CaCl₂, 8 μ g/ml micrococcal nuclease, 14 mM EGTA, 85 μ g/ml calf liver tRNA, 1 mM dithiothreitol, 60 μ M of the unlabelled amino acids, 1 mCi/ml [³⁵S]methionine (Amersham) and 50–100 μ g/ml RNA. Translation was at 30°C for 90 min.

Wheat germ. Translations in the wheat germ extract contained 30% (v/v) wheat germ extract, 20 mM Hepes–KOH, pH 7.5, 120 mM potassium acetate, 3 mM magnesium acetate, 0.5 mM dithiothreitol, 95 μ M unlabelled amino acids, 2.5 mM ATP, 0.4 mM GTP, 10 mM creatine phosphate, 100 μ g/ml creatine kinase, 50 μ g/ml calf liver tRNA, 1 mCi/ml [³⁵S]methionine and 50–100 μ g/ml RNA. Translation was at 30°C for 1 h.

These conditions were found to allow efficient translation of all the RNAs tested. All translation products were analysed on SDS–polyacrylamide gels as described by Laemmli (1970). In some cases, translation products were quantified as described by Godefroy-Colburn *et al.* (1985) or by microdensitometry.

Immunoprecipitation of translation products

The procedure of Hellmann *et al.* (1983) was followed with minor modifications. The translation mixture (10 μ l) was boiled for 3 min in 1% SDS and 1% β -mercaptoethanol, and then diluted with 90 μ l of TBS–detergent mix (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 0.5% deoxycholate and 1% Triton X-100) containing 10 mg/ml BSA. Antiserum or preimmune serum (5 μ l) were then added and the mixture incubated for 1 h at room temperature followed by the addition of 20 μ l of a suspension of *Staphylococcus aureus* cells (Pansorbin, Calbiochem). The mixture was incubated for 20 min, centrifuged for 2 min at 5000 r.p.m. and the pellet resuspended in 200 μ l of TBS–detergent mix. This procedure was repeated five times and the final pellet dissociated by boiling for 3 min in 1% SDS, 5% β -mercaptoethanol before PAGE.

Activity gel analysis

To detect DNA polymerase activities in plant extracts (prepared as described in Pfeiffer *et al.*, 1984) and translation products, proteins were analysed on polyacrylamide gels (Laemmli, 1970) containing 50 μ g/ml activated DNA copolymerized with the gel (Spanos and Hübscher, 1982). Proteins were renatured *in situ* after electrophoresis by a series of washes in 50 mM Tris–HCl, pH 7.5, with either 0.1% β -mercaptoethanol or 1 mM dithiothreitol, over 5–6 h at room temperature. The gel was then incubated overnight in 2 volumes of 50 mM Tris–HCl, pH 7.5, 50 mM KCl, 10 mM MgCl₂, 1 mM dithiothreitol, 100 μ M each of dATP, dGTP and dTTP and 50 μ Ci of [³²P]dCTP. Unincorporated dCTP was removed by several washes in 5% trichloroacetic acid (TCA) at room temperature. The gel was then stained and dried for autoradiography. Dark bands of TCA-insoluble, incorporated radioactivity show the location on the gel of proteins with DNA polymerase activity.

Detection of RNase H activity

RNase H activity was also assayed on activity gels containing a [³²P]RNA–unlabelled DNA hybrid. This was produced by random transcription of 100 μ g of single-stranded M13 DNA with 10 units of *E. coli* RNA polymerase in a 200 μ l reaction mixture containing 50 mM Tris–HCl, pH 7.8, 100 mM KCl, 8 mM MgCl₂, 2 mM DTT, 200 μ M ATP, GTP and CTP and 100 μ Ci [³²P]UTP for 30 min at 37°C. The products were then extracted with phenol and ethanol precipitated, before treatment with RNase A in 2 $\times$ SSC to digest any RNA not hybridized to DNA.

After electrophoresis of nuclear extracts (Pfeiffer *et al.*, 1984) and translation products, the gel was incubated at 37°C in several changes of renaturation/reaction buffer (50 mM Tris–HCl, pH 7.8, 50 mM KCl, 10 mM MgCl₂) over a period of 16 h. The gel was then stained with Coomassie Blue and dried before autoradiography. Proteins with RNase H activity digested the labelled RNA, and the resulting bleaching of the dark background revealed their position on the autoradiogram.

Acknowledgements

We are grateful to Drs Louise Givord, Hubert Guilley, Gérard Jonard, Ken Richards, Véronique Ziegler and Maciej Pietrzak for valuable discussions and gifts of antiserum, Witold Filipowicz and Jan Hofsteenge for critically reading the manuscript and to Hanny Schmid for expert technical assistance. Drs T. Jacks and H. Varmus kindly provided the plasmid pGP, and Drs Thérèse Godefroy-Colburn and Monique Pinck the wheat germ extract. K.G. was supported by an EMBO long-term fellowship.

References

- Ahlquist, P., Dasgupta, R. and Kaesberg, P. (1984) *J. Mol. Biol.*, **172**, 369–383.
- Al Ani, R., Pfeiffer, P. and Lebeurier, G. (1979) *Virology*, **93**, 188–197.
- Al Ani, R., Pfeiffer, P., Whitechurch, O., Lesot, A., Lebeurier, G. and Hirth, L. (1980) *Ann. Virol. (Inst. Pasteur)*, **131E**, 33–53.
- Bonneville, J.M., Fütterer, J., Gordon, K., Hohn, T., Martinez-Izquierdo, J., Pfeiffer, P. and Pietrzak, M. (1987) *UCLA Symp. New Series*, **48**, 267–294.
- Brisson, N., Paszkowski, J., Penswick, J.R., Gronenborn, B., Potrykus, I. and Hohn, T. (1984) *Nature*, **310**, 511–516.
- Clare, J. and Farabaugh, P. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 2829–2833.
- Condit, C., Hagen, T.J., McKnight, T.D. and Meagher, R.B. (1983) *Gene*, **25**, 101–108.
- Copeland, T.D., Gerad, G.F., Hixson, C.W. and Oroszlan, S. (1985) *Virology*, **143**, 676–679.
- Covey, S.N. and Hull, R. (1981) *Virology*, **111**, 463–474.
- Dixon, L. and Hohn, T. (1984) *EMBO J.*, **3**, 2731–2736.
- Dixon, L.K., Koenig, I. and Hohn, T. (1983) *Gene*, **25**, 189–199.
- Farmerie, W.G., Loeb, D.D., Casavant, N.C., Hutchinson, C.A. III, Edgell, M.H. and Swanstrom, R. (1987) *Science*, **236**, 305–308.
- Franck, A., Guilley, H., Jonard, G., Richards, K. and Hirth, L. (1980) *Cell*, **21**, 285–294.
- French, R., Janda, M. and Ahlquist, P. (1986) *Science*, **231**, 1294–1297.
- Fütterer, J. and Hohn, T. (1987) *Trends Biochem. Sci.*, **12**, 92–95.
- Gardner, R.C., Howarth, A.J., Hahn, P., Brown-Luedi, M., Shepherd, R.J. and Messing, J. (1981) *Nucleic Acids Res.*, **9**, 2871–2888.
- Giband, M., Mesnard, J.M. and Lebeurier, G. (1986) *EMBO J.*, **5**, 2433–2438.
- Givord, L., Xiong, C., Giband, M., Koenig, I., Hohn, T., Lebeurier, G. and Hirth, L. (1984) *EMBO J.*, **3**, 1423–1427.
- Godefroy-Colburn, T., Thivent, C. and Pinck, L. (1985) *Eur. J. Biochem.*, **147**, 541–548.
- Hahn, P. and Shepherd, R.J. (1982) *Virology*, **116**, 480–488.
- Harker, C.L., Mullineaux, P.M., Bryant, J.A. and Maule, A.J. (1987) *Plant. Mol. Biol.*, **8**, 275–287.
- Hellmann, G.M., Thornbury, D.W., Hiebert, E., Shaw, J.G., Pirone, T.P. and Rhoads, R.E. (1983) *Virology*, **124**, 434–444.
- Hohn, T., Hohn, B., Lesot, A. and Lebeurier, G. (1980) *Gene*, **11**, 21–31.
- Hohn, T., Hohn, B. and Pfeiffer, P. (1985) *Trends Biochem. Sci.*, **10**, 205–209.
- Hope, I.A. and Struhl, K. (1985) *Cell*, **43**, 177–188.
- Hull, R. and Covey, S.N. (1986) *J. Gen. Virol.*, **67**, 1751–1758.
- Hull, R., Shepherd, R.J. and Harvey, J.D. (1976) *J. Gen. Virol.*, **31**, 93–100.
- Jacks, T., Townsley, K., Varmus, H.E. and Majors, J. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 4298–4303.
- Jacks, T. and Varmus, H.E. (1985) *Science*, **230**, 1237–1242.
- Laemmli, U.K. (1970) *Nature*, **227**, 680–685.
- Lütcke, H.A., Chow, K.C., Mickel, F.S., Moss, K.A., Kern, H.F. and Scheele, G.A. (1987) *EMBO J.*, **6**, 43–48.
- Maniatis, T., Fritsch, E.F. and Sambrook, J. (eds) (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Martinez-Izquierdo, J. and Hohn, T. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 1824–1828.
- Martinez-Izquierdo, J.A., Fütterer, J. and Hohn, T. (1987) *Virology*, **160**, 527–530.
- Mason, W.S., Taylor, J.M. and Hull, R. (1987) *Adv. Virus Res.*, **32**, 35–96.
- Mellor, J., Fulton, S.M., Dobson, M.J., Wilson, M., Kingsman, S.M. and Kingsman, A.J. (1985) *Nature*, **313**, 243–246.
- Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. and Green, M.R. (1984) *Nucleic Acids Res.*, **12**, 7035–7056.
- Menissier, J., Laquel, P., Lebeurier, G. and Hirth, L. (1984) *Nucleic Acids Res.*, **12**, 8769–8778.
- Menissier-de Murcia, J., Geldreich, A. and Lebeurier, G. (1986) *J. Gen. Virol.*, **67**, 1885–1891.
- Müller, F., Brühl, K.H., Freidel, K., Kowallik, K.V. and Ciriacy, M. (1987) *Mol. Gen. Genet.*, **207**, 421–429.
- Odell, J.T. and Howell, S.H. (1980) *Virology*, **102**, 349–359.
- Peabody, D.S. and Berg, P. (1986) *Mol. Cell. Biol.*, **6**, 2695–2703.
- Pelham, H.R.B. and Jackson, R.J. (1976) *Eur. J. Biochem.*, **67**, 247–256.
- Pfeiffer, P., Laquel, P. and Hohn, T. (1984) *Plant Mol. Biol.*, **3**, 261–270.
- Pietrzak, M. and Hohn, T. (1987) *Virus Genes*, **1**, 83–96.
- Plant, A.L., Covey, S.N. and Grierson, D. (1985) *Nucleic Acids Res.*, **13**, 8305–8321.
- Rezaian, M.A., Williams, R.H.V., Gordon, K.H.J., Gould, A.R. and Symons, R.H. (1984) *Eur. J. Biochem.*, **143**, 277–284.
- Sieg, K. and Gronenborn, B. (1982) *Abstr. NATO Adv. Stud. Inst.*, 1982, pp. 154–154.
- Spanos, A. and Hübscher, U. (1982) *Methods Enzymol.*, **91**, 263–277.
- Tabor, S. and Richardson, C.C. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 1074–1078.
- Tanese, N., Roth, M. and Goff, S.P. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 4944–4948.
- Toh, H., Ono, M., Saigo, K. and Miyata, T. (1985) *Nature*, **315**, 691.
- Xiong, C., Muller, S., Lebeurier, G. and Hirth, L. (1982) *EMBO J.*, **1**, 971–976.
- Young, M.J., Daubert, S.D. and Shepherd, R.J. (1987) *Virology*, **158**, 444–446.
- Ziegler, V., Laquel, P., Guilley, H., Richards, K. and Jonard, G. (1985) *Gene*, **36**, 271–279.

Received on October 12, 1987; revised on November 27, 1987