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Detection of a subgenomic mRNA for gene V, the putative reverse transcriptase gene of cauliflower mosaic virus

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ABSTRACT

Polypeptides synthesized in vitro in rabbit reticulocyte lysates, directed by poly(A)$^+$ RNA isolated from turnip leaves infected with cauliflower mosaic virus (CaMV), were analysed by polyacrylamide gel electrophoresis. Following translation of virus-specific RNA purified by hybrid-selection using CaMV DNA immobilized on DBM papers, the polypeptides observed included the viral gene VI inclusion body protein P62, and a larger product, P75, together with several smaller polypeptides. By translating RNA hybrid-selected with restriction fragments encompassing the CaMV genome, a mRNA for P75 has been mapped to gene V. These results, together with sucrose gradient ultracentrifugation studies, suggest that a CaMV gene V mRNA is a sub-genomic transcript of approximately 2.5Kb and 22S. The expression strategy of the CaMV genome is discussed in the light of our findings.

INTRODUCTION

Cauliflower mosaic virus (CaMV) is the type member of an unusual group of plant viruses containing circular double-stranded DNA (for review see (1)). The primary DNA sequence of CaMV (2-4) reveals the presence of eight long open reading frames (ORFs), all of which are coded by the plus strand. The ORFs alternate in reading frame and closely abutt or overlap, except for a large intergenic region (~700bp) between ORFs VI and VII and two smaller ones between VII and I (~ 60bp) and V and VI (~ 100bp) (see Fig. 1). Protein products for ORFs II, III, IV, V and VI have been detected in infected plants (2,5-9, 39,40). ORF VI codes for a polypeptide of 62K mol.wt. (P62), a major constituent of the inclusion bodies found in infected cells (6,10,11) and ORF II codes for a factor (P18) involved in the aphid transmission of CaMV (5,8). The product from ORF IV is the precursor to the coat protein (2,9). Amino acid sequence

homologies between ORF V of CaMV and the pol gene products of animal retroviruses strongly suggest that ORF V might code for a protein involved in the replication of CaMV DNA by reverse transcription (12,13,14). Moreover, an 80K mol. wt. polypeptide product of ORF V has been detected in extracts from CaMV infected plants (39).

The gene expression strategy adopted by CaMV has not yet been fully elucidated. It has been shown that viral DNA detected in the nucleus as mini-chromosomes (15) serves as a template for transcription by RNA polymerase II (16,17). Furthermore, two major polyadenylated RNA species have been found in infected cells (10,11). The larger species (35S RNA) is a transcript of the complete viral genome with a terminally redundant sequence of 180 nucleotides (26). This RNA has been proposed as the template for reverse transcription during replication of the viral genome (18,19). The other major species (19S RNA) is transcribed from ORF VI and is the mRNA for the inclusion body protein P62 (10,11). In addition, minor CaMV RNAs of 8Kb and 0.6Kb have been described by Guilley et al. (20), while Condit et al. (21) have reported the detection of transcripts of 4.9, 4.5, 4.3 and 1.8Kb. However of all these transcripts described above, only one, the 19S RNA, has been shown to have messenger activity in vitro and to code for a product in vivo (10).

There is evidence that the expression of ORFs VII and I (22) and I-III (23,24) is linked by translation from a polycistronic mRNA (possibly 35S RNA) by a "relay race" mechanism in which the ribosome, after passing a termination codon, is thought to reinitiate protein synthesis at the nearest downstream AUG codon. However, little is known about the expression of ORF IV and V. In this paper we describe the detection by in vitro translation of mRNAs coding for novel CaMV polypeptides and provide evidence that the putative reverse transcriptase gene is expressed via a subgenomic mRNA.

MATERIALS AND METHODS

Virus

CaMV isolate Cabb B-JI was propagated in turnip (Brassica campestris L. cv. Just Right). 12 day old plants were sap-

inoculated with material derived from turnip leaves infected with the excised insert of pMD324, a full length DNA clone of CaMV Cabb B-JI in pAT153 (25).

RNA preparation

RNA was prepared from CaMV infected turnip leaves 22 days post inoculation as described by Covey and Hull (11). Poly(A)$^+$ RNA was purified by fractionating samples of heat denatured (75°C for 2 min) total RNA with two passages through oligo(dT)-cellulose as described by Covey et al. (26). Poly(A)$^+$ RNA was precipitated with ethanol plus 20µg/ml calf liver tRNA as carrier (Boehringer).

In vitro translation

Messenger RNA was translated in vitro in rabbit reticulocyte lysates obtained either from Amersham International PLC or prepared according to Hunt and Jackson (27), and rendered messenger dependent by the method of Pelham and Jackson (28). Translation mixtures typically contained 16µl lysate, 0.1M creatine phosphate, 110mM potassium chloride, 1.5-2.0mM magnesium chloride, 1mM of each amino acid excepting methionine, 10-13µCi [^{35}S] methionine, (New England Nuclear or Amersham International PLC) 20µg whole cell RNA or 1µg poly(A)$^+$ RNA and were incubated at 30°C for 60 min.

Gel electrophoresis

Proteins were fractionated on SDS/linear gradient polyacrylamide (7.5-25% or 7.5-20%), bisacrylamide (0.2-0.125% or 0.2-0.1%), slab gels with a 4.5% acrylamide, 0.009% bisacrylamide stacking gel using the buffer system of Laemmli (29). Protein samples were denatured at 100°C in 4 vols sample buffer (2.5mM Tris-HCl, pH 8.0, 2% SDS (w/v), 5% 2-mercaptoethanol, 25mM EDTA, 15% sucrose and 0.5% bromophenol blue) before electrophoresis at 60V, 20mA, until the tracking dye reached the bottom of the gel. [^{35}S] methionine labelled polypeptides and [^{14}C] labelled marker proteins (Amersham International PLC) were detected by fluorography (30).

Hybrid-selection of CaMV poly(A)$^+$ RNA

The following cloned DNAs were prepared by the method of Birnboim and Doly (31) for binding to DBM paper (32): pAT153; pCa24 (a full length infectious clone of CaMV Cabb B-JI inserted

at the SalI site of pAT153); CaMV BglII fragments -a,-b,-c,-d and -e cloned into pKC7 (pCa2,-8,-7,-5 and -9 respectively); EcoRI fragment -c cloned into pMB9 (pCa20); HindIII fragments -a,-b,-c and -d cloned into M13mp9 (pBJI070,-073,-079 and -065 respectively); and BglII- EcoRI fragments cloned into M13mp8 (pBJI V and pBJI VI) (see Fig. 1). pAT153 was linearised at the SalI site, while the cloned CaMV fragments were excised at their respective cloning sites with the appropriate restriction enzyme. CaMV sequences from pBJI070,-073,-079,-065, pCa9, pBJI V and pBJI VI were purified by centrifugation in a 15-30% sucrose gradient (in 50mM Tris-HCl, pH 7.5, 1mM EDTA) at 36K rpm in a Beckman SW40 rotor for 20 hrs. DNA was denatured before binding to DBM paper as described by Christophe et al. (33). 20-25µg DNA was incubated with each $1cm^2$ DBM disc. For the purification of virus-specific transcripts, 10µg poly(A)$^+$ RNA was incubated with DBM discs in hybridization buffer containing 20mM PIPES-NaOH, pH 6.4, 1mM EDTA, 0.6M NaCl, 0.2% SDS, 50% formamide at 42°C for 18 hrs. After hybridization, DBM discs were thoroughly washed in several changes of hybridization buffer at 45°C for 1.5 hrs. Hybridized RNA was eluted from DNA-DBM paper by incubation in 10mM Tris-HCl pH 7.8, 1mM EDTA at 75°C for 3 min. Eluted RNA was precipitated with ethanol plus 20µg/ml calf liver tRNA as carrier and then dissolved in sterile distilled water for translation in vitro.

Sucrose gradient fractionation of virus-specific RNA

Virus-specific RNA was hybrid-selected with pCa24 from 60µg of total cell poly(A)$^+$ RNA as described above. It was then heat-denatured at 70°C for 2 min and fractionated in a 7-25% sucrose gradient (in 0.15M LiCl, 5mM EDTA, 0.1% SDS, 50mM Tris-HCl, pH 8.0). Gradients were centrifuged at 22,000 rpm for 16-18 hr at 10°C in a Beckman SW27 rotor. Following centrifugation, 0.5ml fractions were collected and the RNA recovered by precipitation with ethanol plus 10µg calf liver tRNA as carrier.

RESULTS

A physical map of the CaMV Cabb B-JI genome showing features relevant to this paper, is presented in Fig. 1. Sequencing of isolate Cabb B-JI (J. Stanley, personal communication) has shown

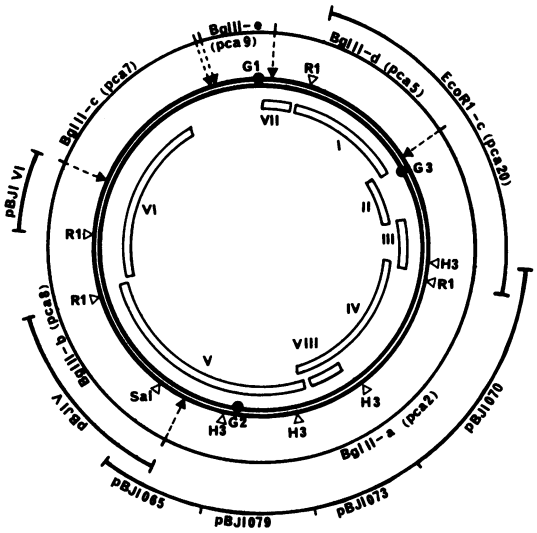

FIGURE 1. Physical map of the CaMV genome. The thick double line represents the double-stranded DNA showing the three discontinuities, G1 in the DNA (-) strand, G2 and G3 in the DNA (+) strand. Restriction enzyme sites: R1 = EcoRI, H3 = HindIII and Sal = SalGI are shown (▽). BglII sites are shown (▼). The outer circle and segments are cloned restriction fragments used in the hybrid-selection experiments. The arrangement of open reading frames I-VIII is shown.

that it has a similar pattern of ORFs to the previously published sequences of other CaMV isolates (2,3,4).

In vitro translation of RNA

When total RNA, extracted from leaves systemically infected with CaMV, was translated in vitro in rabbit reticulocyte lysates, a major translation product was the viral inclusion body protein P62 (11) (Fig. 2A). This polypeptide was absent when RNA extracted from healthy leaves was translated in vitro (Fig. 2B). Other major differences included polypeptides of 75K and 82K present only among polypeptides synthesized when lysate was programmed with RNA isolated from infected leaves (Fig. 2A) and a polypeptide of approximately 20K observed only among those polypeptides synthesized in vitro by RNA isolated from healthy tissue (Fig. 2B). A similar pattern of translation products was observed when poly(A)$^+$ RNA from infected (Fig 2C) and healthy

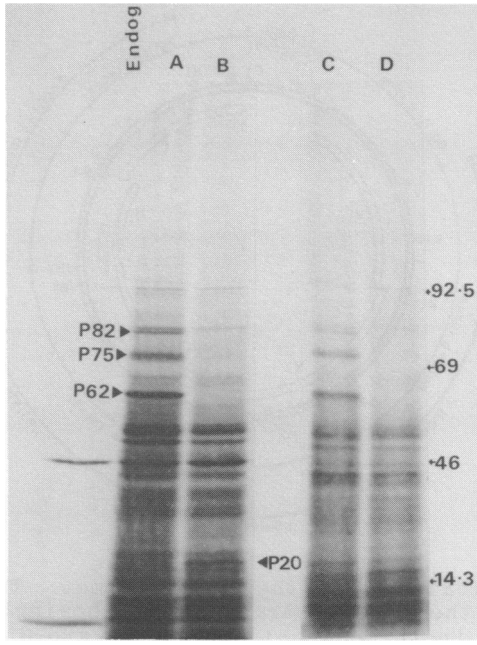

FIGURE 2. Fluorogram of SDS/linear 7.5-25% gradient polyacrylamide gel-fractionated [^{35}S] methionine-labelled proteins synthesized in vitro in rabbit reticulocyte lysates programmed with no RNA(Endog), 20µg whole cell RNA from CaMV infected tissue (A) and healthy tissue (B) and 1µg poly(A)$^+$ RNA from CaMV infected tissue (C) and healthy tissue (D). The size of molecular weight markers (x10^3) are shown. The proteins indicated (▼) are discussed in the text.

tissue (Fig. 2D), isolated using oligo(dT)-cellulose, was translated in vitro.

Translation of virus-specific RNA

To determine which of the several novel polypeptides detected, when RNA from CaMV infected tissue was translated in vitro, were encoded by the viral genome, poly(A)$^+$ RNA isolated from infected leaves was hybrid-selected with full-length CaMV DNA (pCa24) covalently bound to DBM paper. When the virus-specific transcripts were eluted and translated in vitro the majority of the [^{35}S] methionine was incorporated into P62, the inclusion body protein (Fig. 3B). However, other polypeptides observed included those of 80K, 75K, 57K, 54K and 38K mol.wt. No labelled products were observed when poly(A)$^+$ RNA from healthy tissue was

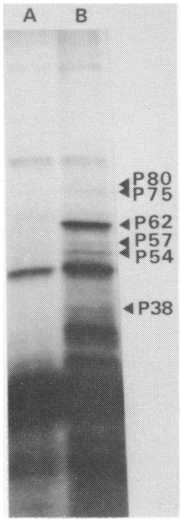

FIGURE 3. In vitro translation products directed by virus specific poly $(A)^+$ RNA from healthy tissue (A) and CaMV-infected tissue (B) after hybrid-selection with pCa24 DNA and fractionation in a 7.5-25% polyacrylamide gradient gel. The indicated (▼) proteins are discussed in the text. Only those polypeptides endogenous to the lysate were present in A.

hybrid-selected with pCa24 DNA and translated in vitro (Fig. 3A) other than those endogenous to the lysate (see Fig. 5). Similarly, no labelled products were observed when poly$(A)^+$ RNA from infected tissue was hybrid-selected by pAT153 DNA bound to DBM paper and translated in vitro (data not shown).

Mapping mRNAs for P75 and other polypeptides by hybrid-selection

To map the mRNAs for the polypeptides observed by hybrid-selection, poly$(A)^+$ RNA isolated from infected leaf tissue was hybridised to a number of individual restriction fragments of the CaMV genome (Fig.1) covalently attached to DBM paper, and the selected mRNA was translated in vitro.

The initial mapping was performed using the large BglII clones pCa5, 2, 8 and 7 encompassing all of the viral ORFs (see Fig. 1). Messenger activity for P75 was hybrid-selected by the genome length clone pCa24 (Fig. 4B), pCa2 (Fig. 4C) containing 3' sequences from ORF I, sequences from ORFs II, III, IV and VIII and the 5' portion of ORF V, and by pCa8 (Fig. 4F) containing parts of ORFs V and VI but not by pCa5 (Fig. 4D) containing ORF VII and most of ORF I. However, P75 was hybrid-selected for faintly by pCa7 (Fig. 4E) which contains ORF VI and the termini of the replication template 35S RNA. In addition to P75, several smaller polypeptides were also observed following translation of

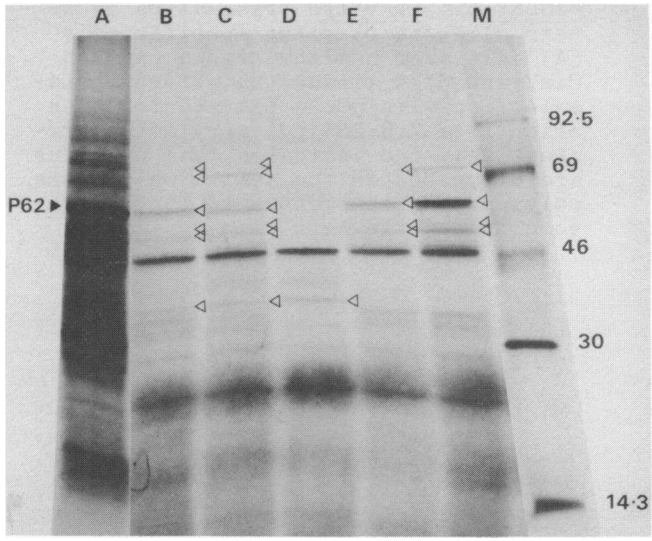

FIGURE 4. In vitro translation products synthesized when lysate was programmed with virus-specific poly(A)$^+$ RNA following hybridization with pCa24 (B), pCa2 (C), pCa5 (D), pCa7 (E) and pCa8 DNA (F). Polypeptides were fractionated on a 7.5-25% gradient polyacrylamide gel. (A) in vitro translation products directed by 20µg whole cell RNA from infected tissue. Molecular weight markers (x10^3) (M) are shown. The indicated proteins ◁ , P80, P75, P62, P60, P57, P54 and P38 in order of increasing mobility are discussed in the text.

RNA hybrid-selected by these clones. Among these, P62 was selected for only by those clones containing ORF VI (Fig. 4), and a slightly smaller polypeptide P60, distinct, but not always resolved from P62, was selected by pCa2, pCa8 and pCa7 (Fig. 4C,E, F). When whole cell RNA from Cabb B-JI infected tissue was translated in vitro (Fig. 4A) a slightly smaller polypeptide, P73 was observed in addition to P75.

Finer mapping was achieved using a series of smaller clones for hybrid selection (Fig. 5). P75 messenger activity was selected by clones pBJI079, pBJI065 and pBJI V (Fig. 5D-F) which are internal to ORF V and by clone pBJI073 (Fig. 5C) which contains 5' sequences from ORF V, sequences from ORF VIII and 3' portions of ORF IV. Clone pCa20 encompassing ORFs I, II, III and the 5' end of IV did not select for P75 (Fig. 5A). However, weak selection for P75 was observed with pBJI070 containing sequences from

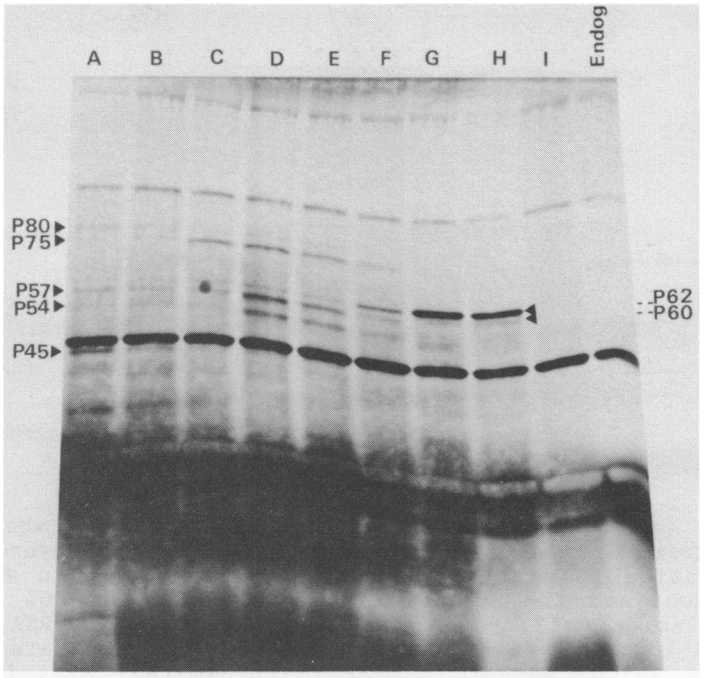

FIGURE 5. In vitro translation products synthesized when lysate was programmed with virus-specific poly(A)$^+$ RNA following hybrid selection with pCa20 (A), pBJ1070 (B), pBJ1073 (C), pBJ1079 (D), pBJ1065 (E), pBJ1 V (F), pBJ1 VI (G), pCa7 (H) and pCa9 DNA (I) and with no RNA (Endog). The products were fractionated on a 7-20% gradient polyacrylamide gel. The proteins indicated (▼) are discussed in the text.

ORF IV, pBJI VI containing sequences from ORF VI and pCa7 containing sequences from ORF VI and part of the large intergenic region (Fig. 5B,G,H). Even so, a consistent feature of all our experiments was that strong hybrid-selection of messenger activity for P75 occurred only with those clones containing portions of ORF V. Clones containing ORF VI and the 3' portion of ORF IV, although selecting for P75, did so only at a level one-tenth or less that of clones containing sequences specific for ORF V. Clone pCa9 containing the large intergenic region of CaMV DNA did not hybrid-select for any polypeptides (Fig. 5I). P62 messenger activity was hybrid-selected for by pBJI V (Fig. 5F) even though this clone is specific to ORF V, containing no

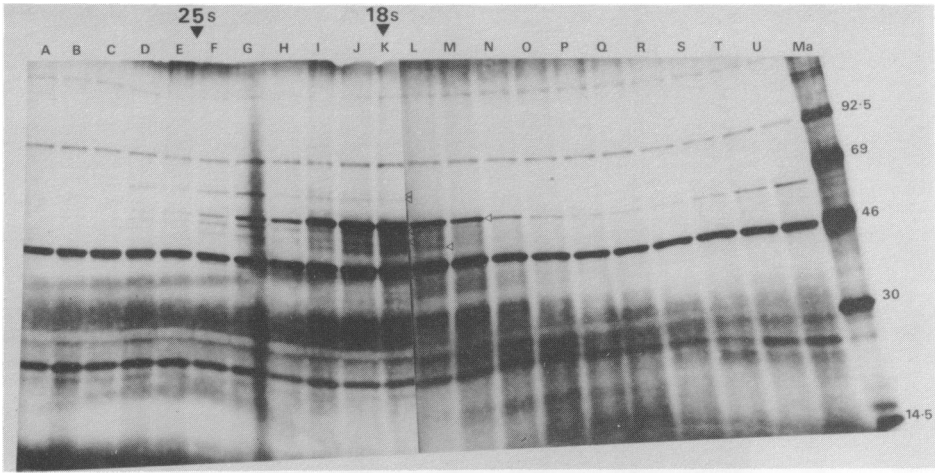

FIGURE 6 In vitro translation products synthesized when lysate was programmed with CaMV-specific poly(A)$^+$ RNA recovered after sedimentation in a sucrose gradient as described in Materials and Methods and the products fractionated on a 7-20% gradient polyacrylamide gel (A-U). The position of plant rRNA markers (25S and 18S) run in a parallel gradient are indicated above. Molecular weight markers (x10^3) are shown (M). ◁ Indicated proteins P80, P75, P62, P57, P54, P52 and P45 in order of increasing mobility are discussed in the text.

sequences from ORF VI. Clones containing ORFs VII, I, II, III and the 5' end of IV (e.g. pCa5 and pCa20; Fig. 4D and 5A) did not select for P75 suggesting that the weak selection for P75 by clones adjacent to ORF V was not the result of low-level non-specific binding of RNA to DBM papers.

These experiments also provided some preliminary mapping data of messenger activity for several other polypeptides including P80, P60, P57, P54, P45 and P38 (Fig. 5) and this is summarised in Table 1.

Sucrose gradient ultracentrifugation of hybrid-selected CaMV RNA

The results in the preceding section provide evidence that the messenger activity for P75 and other polypeptides resides with CaMV transcripts of subgenomic size. To confirm these observations, hybrid-selected CaMV poly(A)$^+$ RNA was heat-denatured and sedimented by sucrose gradient ultracentrifugation. Fractions were collected and the recovered RNA translated in vitro and the resulting polypeptides separated on polyacrylamide gels (Fig. 6). Sedimentation values of CaMV messenger activity were

compared with those of 25S and 18S plant ribosomal RNAs run on a parallel gradient. The ORF VI mRNA for P62 sedimented as a broad peak consistent with previously published data suggesting that this mRNA was 19S (10,11). The messenger activity for P75 overlapped that of P62 but sedimented more rapidly than 19S RNA at approximately 22S (Fig. 6). We detected no messenger activity for P75 or any other polypeptides in the 35S region of the gradient (data not shown). These results confirm our conclusion from hybrid-selection experiments that P75 messenger activity resides within a subgenomic CaMV transcript or transcripts and not the full-length terminally redundant 35S transcript. A number of smaller viral-specific polypeptides present when virus-specific RNA was translated in vitro were also observed following translation of sucrose gradient fractions (Fig. 6).

DISCUSSION

In this paper we have presented evidence that ORF V, the putative reverse transcriptase gene of CaMV, which is sufficiently long to encode a 79K mol.wt. polypeptide (2), is expressed via a subgenomic polyadenylated RNA. This conclusion was drawn from the direct detection of messenger activity for a polypeptide P75 (our size estimate from polyacrylamide gels) by in vitro translation using rabbit reticulocyte lysates. Messenger activity for P75 was hybrid-selected by a full-length CaMV DNA clone, pCa24 bound to DBM papers (Fig. 3B) demonstrating that it was a virus-encoded polypeptide. Covey and Hull (11) have detected a similar sized polypeptide synthesized by in vitro translation of CaMV-specific poly(A)$^+$ RNA. Careful comparisons of the translation products of cell total RNA and hybrid-selected RNA revealed that in addition to the CaMV-specific P75 a slightly smaller polypeptide (P73) was present among translation products of total RNA from CaMV-infected plants (Fig. 4A,B). It is possible that these polypeptides were not resolved when whole cell RNA from CaMV infected plants was translated in vitro and the products fractionated on polyacrylamide gels (Fig. 2A) and migrated together with P75. Messenger activity for P73 was not hybrid-selected by CaMV DNA indicating that this must represent a host gene that was activated during virus infection. Similar-

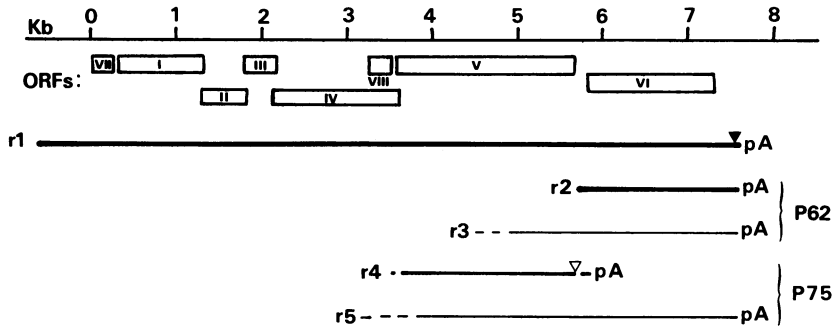

FIGURE 7. CaMV polyadenylated RNAs aligned with the viral open reading frames (ORFs): r1, genome-length 35S RNA; r2, 19S RNA transcript for ORF VI; messenger activities as determined in this paper for P62 (r2+r3); major messenger activity (r4) and minor messenger activity (r5) for P75. Putative poly(A) signal sequences: ▼ AATAAA; ▽ AATAAG.

ly, the polypeptide P82 observed on translation of total RNA (Fig. 2) is probably the product of a host gene and distinct from P80 found in CaMV hybrid-selected RNA translation products (Figs. 3-6).

The messenger activity for P75 was mapped by hybrid-selection experiments using a series of CaMV DNA cloned restriction fragments bound to DBM papers. Only those clones containing sequences specific for ORF V strongly selected for P75 (Fig. 4 and 5). However, low levels of selection for P75 were observed with clones covering ORF VI and extending into (but not at the 5' end of) ORF IV. Detection of subgenomic CaMV transcripts (other than the gene VI, 19S RNA) has been attempted by Northern blot hybridizations (Covey and Turner, unpublished results). This study revealed a heterogeneous sized population of molecules extending around the entire genome but 3' coterminal with the replication template 35S RNA and the gene VI 19S mRNA (see Fig. 7). These heterogeneous RNAs may be related to the replication of CaMV by reverse transcription, however, it has not been possible to distinguish true mRNAs from this population of molecules by Northern blotting. The low level of messenger activity for P75 selected by clones containing sequences specific to ORF IV and VI (Figs. 4 and 5) might have resulted from fortuitous translation of these 3' coterminal fragments that over-

lap ORF V and contain sequences for ORFs IV and VI. In support of this view, a clone, pBJI V, which was internal to the 3' half of ORF V also selected for P62, when it is known that the 19S transcript for P62 is transcribed from a DNA sequence downstream of this clone (26) thus indicating that heterogeneous 3' co-terminal RNA fragments extending into ORF V can be translated in vitro to produce P62 (Fig. 5F).

The 3' end of the gene V mRNA must be located close to the 3' end of ORF V since strong hybrid selection for P75 was not obtained when adjacent clones containing sequences from ORF VI were used (e.g. pBJI VI and pCa7). It is not clear how the 3'-end of this RNA is generated as its fine structure has not yet been determined. Since the RNA has strong affinity for oligo (dT)-cellulose it is probably polyadenylated. Inspection of the DNA sequence in this region of the CaMV genome reveals the presence of a possible poly(A) signal sequence AATAAG, some 18 nucleotides upstream of the ORF V termination codon. Similarly, a major 5'-end for the gene V mRNA must be located close to the 5'-end of ORF V or ORF VIII (both are in the same reading frame; Fig. 1) since strong hybrid-selection for P75 was not observed with an adjacent clone (pBJI070) just upstream of this region. Also, clones covering ORFs VII, I, II and the 5' end of ORF IV did not hybrid-select for P75 (Figs. 4 and 5); which indicates that the 5' end of the gene V mRNA must lie downstream of the DNA sequences covered by these clones. We have not found sequences reminiscent of eukaryotic promoters (34) immediately upstream of ORF V or ORF VIII that might suggest that transcription is initiated within this region. However, the possibility that the 5' end of 35S RNA is spliced onto the gene V mRNA cannot be discounted. The leader of the 35S RNA contains a single, almost perfect, splice donor consensus sequence 445 nucleotides from its 5' end (38). A small clone containing this splice donor sequence (pCa9) did not select for P75 messenger activity (Fig. 5I), However a smaller splice might occur containing only sequences from the 5' end of the 35S RNA which are not contained within pCa9 but covered by the upstream clone pCa7. Indeed, this might in part explain the selection for P75 by this clone. (Fig. 4E,5H).

From the hybrid-selection experiments we conclude that a major gene V mRNA of approximately 2.5Kb is transcribed from ORF V and that a sub-population of heterogeneous molecules overlapping ORF V might also be translated to produce P75 (Fig. 7). Sucrose gradient ultracentrifugation confirmed that the messenger activity for P75 was subgenomic, although heterogenous in size, and indicated that it was approximately 22S (Fig. 6).

There is evidence that expression of CaMV ORFs VII, I, II and III is linked by a "relay race" mechanism of translation occurring on a polycistronic mRNA (22-24) and it has been suggested by Hull (41) that folding of 35S RNA could bring ribosomes to internal ORFs. The experiments presented in this paper did not address the question as to whether the 35S RNA is a polycistronic transcript since it is considered unlikely that rabbit reticulocyte lysates could translate or process such a mRNA. Indeed, no messenger activity for any polypeptides was observed when sucrose gradient fractions containing 35S RNA were translated in vitro (results not shown). By analogy with animal retroviruses and eukaryotic retrotransposons which exhibit many similarities to CaMV both in replicative processes and genome organization, it might be expected that translation of CaMV gene IV (coat protein) and gene V (polymerase) is also linked and that a polyprotein would be produced analogous to the retrovirus gag-pol polyprotein (35,36). However, generation of the retrovirus gag-pol fusion protein is a relatively inefficient process (35, 36), and,should this also be the case for a CaMV fusion protein, then specific amplification of gene V could be effected via the generation of the P75 mRNA reported here. Thus, we do not discount the possibility of linked expression for genes IV and V in addition to the generation of subgenomic transcripts.

We also detected messenger activity for several other CaMV polypeptides (see Table 1) of unknown function, two of which, P57 and P54 had messenger activities coinciding with that for P75. These polypeptides might be generated from P75 by a virus-coded protease activity since there exists within gene V a region with amino acid sequence homology with retrovirus protease thought to be involved in processing polyproteins (13). Messenger activity for P45 was hybrid-selected by pCa20 and pBJI070,

TABLE 1

Polypeptides synthesised *in vitro* following hybrid-selection with cloned CaMV restriction fragments

Clone	ORF covered	Polypeptides hybrid selected							
		P80	P75	P62	P60	P57	P54	P45	P38
BglII-a (pCa2)	I,II,III, IV,V	√	√	-	√	√	√	-	√
BglII-b (pCa8)	V,VI	-	√	√	√	√	√	-	-
BglII-c (pCa7)	VI	-	F	√√	F	F	F	-	-
BglII-d (pCa5)	VII,I	-	-	-	-	-	-	-	√
BglII-e (pCa9)	Large intergenic	-	-	-	-	-	-	-	-
EcoRI-c (pCa20)	I,II,III,IV	√	-	-	-	F	F	√	√
pBJ1070	IV	√	F	-	-	F	F	√	-
pBJ1073	IV,V	√	√	-	-	F	F	-	-
pBJ1079	V	√	√	-	-	√	√	-	-
pBJ1065	V	√	√	-	-	√	√	-	-
pBJ1V	V	-	√	√	-	√	√	-	-
pBJ1VI	VI	-	F	√√	√	-	-	-	-

√√= Very strong presence; √ = Strong presence; F = Faint presence; - = Absent

containing sequences from ORF I to ORF IV and ORF IV respectively (Fig. 5, Table I), and hence mapped it to ORF IV. P45 is similar in size to that polypeptide (P43) considered to be the mature coat polypeptide product of ORF IV (2,40). Sucrose gradient ultracentrifugation also demonstrated that the messenger activity was subgenomic and sedimented similarly to the 19S RNA (Fig. 6). The P38 messenger activity was hybrid-selected for only by pCa2 and pCa5 (Fig. 4C,D) which contained common sequences only specific for ORF I which is long enough to encode a 38K polypeptide (2). This messenger activity may also result from a specific amplification of gene I, since there is evidence (22, 23,24) that the expression of ORF I is linked with ORFs II and III. In conclusion, we have provided evidence that a subgenomic

mRNA is involved in the expression of CaMV gene V and possibly for genes I and IV as well. Our results suggest that a dual strategy of expression operates in CaMV and that subgenomic mRNAs might be generated to amplify gene products over and above those derived by translation of a proposed (22-24) polycistronic mRNA.

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