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The Cauliflower Mosaic Virus Reverse Transcriptase Is Not Produced by the Mechanism of Ribosomal Frameshifting in *Saccharomyces cerevisiae*

T. WURCH, T. GUIDASCI, A. GELDREICH, G. LEBEURIER,[1] AND J.-M. MESNARD

Institut de Biologie Moléculaire des Plantes du CNRS, Université Louis Pasteur, 12 rue du Général Zimmer, 67084 Strasbourg Cédex, France

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The capsid protein and the reverse transcriptase of cauliflower mosaic virus (CaMV) are encoded by two genes (ORF IV and ORF V) that lie in different translation reading frames. A comparison can be drawn between the synthesis of both CaMV proteins and the fusion protein in a yeast retrotransposon, Ty, resulting from a +1 frameshifting event which fuses two out-of-phase ORFs encoding the structural protein and the reverse transcriptase of Ty. For this reason, we constructed a yeast expression vector containing CaMV ORF VII fused to CaMV ORF III by a fragment of 452 bp including the overlapping region of ORF IV and ORF V, ORF VII and ORF III being used as reporter genes. We characterized two proteins (22 and 50 kDa) synthesized from this plasmid in the yeast expression system. We demonstrated that the 50-kDa polypeptide is not synthesized from a +1 frameshifting event but is probably a dimeric form of the 22-kDa protein. From this result we conclude that the CaMV reverse transcriptase is not produced by a mechanism of ribosomal frameshifting.

Cauliflower mosaic virus (CaMV) has a 8000 bp long circular double-stranded DNA as genetic material (for recent reviews, see (*1, 2*)). The genome contains seven open reading frames (ORFs). They are closely spaced (ORFs I, II, and III) or they overlap (ORFs III, IV, and V). Small intergenomic regions separate ORFs VII and I (60-odd bp) and ORFs V and VI (100-odd bp) while a larger one (about 600 bp) exists between ORFs VI and VII.

The translation strategy of these ORFs has not yet been fully elucidated except for ORF VI which is translated from a 19 S mRNA (*3*). For ORFs VII, I, II, and III it has been suggested that the proteins are synthesized from a 35 S polycistronic RNA, the translation being initiated at the ATG of ORF VII (*4*). Translation of downstream cistrons (ORFs I, II, and III) would be the result of a termination–reinitiation mechanism (*4, 5*).

Little is known, however, about the expression of ORFs IV and V which code for the capsid protein (*6, 7*) and for the reverse transcriptase, respectively (*8, 9*). These ORFs overlap (by 79 bp for strain Cabb-S (*10*)) and ORF V is in the +1 reading frame with respect to ORF IV. Such an organization has been described for the yeast retrotransposon Ty. This transposon possesses two genes TYA and TYB which code for a structural protein and for an enzymatic polypeptide with reverse transcriptase activity, respectively (*11, 12*). TYA and TYB overlap by 38 bp and TYB is in the +1 reading frame with respect to TYA. TYB is expressed as a TYA-TYB fusion protein (*13*). The production of this fusion polypeptide is achieved via a frameshifting event that leads to the translational avoidance of the TYA termination codon and a shift into the TYB reading phase. This expression strategy is analogous to the production of the gag–pol fusion protein in the retroviruses, gag coding for structural proteins and pol coding for the reverse transcriptase. However, in the case of the retroviruses the gag–pol protein is synthesized via −1 frameshift (*14, 15*) or readthrough (*16*) mechanisms.

A CaMV ORF IV–ORF V fusion protein has not been detected either in infected plants (*7, 17*) or in *in vitro* translation systems (*18*). The absence of a fusion polypeptide could be due to the immediate processing of the protein by the proteolytic activity located at the N-terminal part of the ORF V product (*19*). Likewise some factors necessary for a +1 frameshifting event could be absent in the *in vitro* translation systems. For this reason, we decided to study the expression strategy of CaMV ORF IV–ORF V in the yeast system. This system is a powerful tool since it was used with success to demonstrate a +1 frameshift mechanism for the yeast retrotransposon Ty (*13*).

To exclude the possibility that the fusion protein was processed by the CaMV protease we removed a portion of ORF V indispensable for the proteolytic activity (*19*). For this we selected a fragment of 452 bp (from nucleotide 3461 to nucleotide 3913 of strain Cabb-S of CaMV (*10*)). This fragment contains the region necessary for a frameshifting event: ORF IV ends at nucleotide 3670 and ORF V begins at nucleotide 3591. Positive controls for our assay system were provided by

[1] To whom requests for reprints should be addressed.

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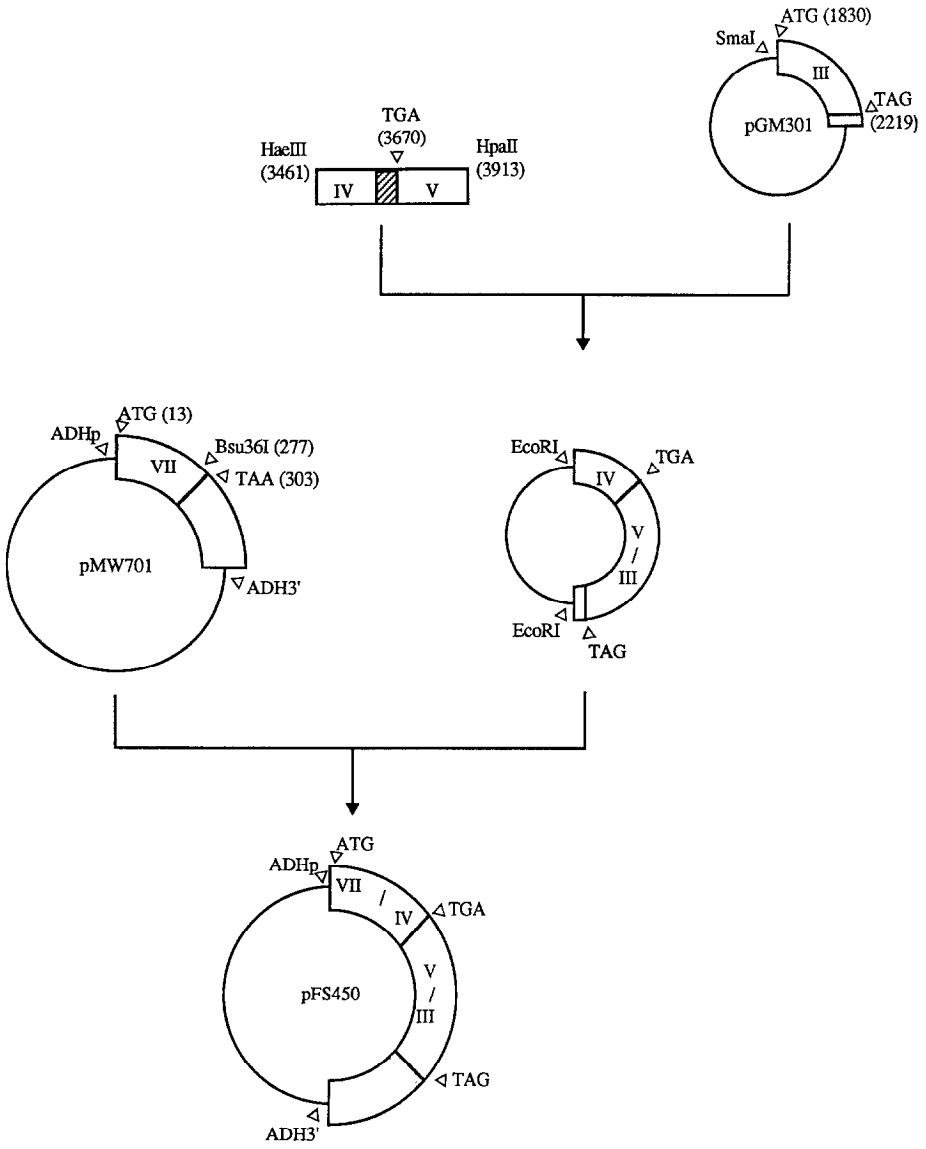

FIG. 1. Construction of plasmid pFS450. Numbers refer to nucleotide positions (*10*) on the CaMV DNA (strain Cabb-S). Boxes represent the CaMV DNA. Thin lines represent the plasmid DNA. The initiation codons of ORF III and ORF VII, the stop codons of ORF III, ORF IV, and ORF VII, the yeast ADH1 gene promoter, the 3′ sequence of yeast ADH1 gene and the location of restriction sites are indicated by arrowheads. CaMV DNA was digested with *Hae*III and *Hpa*II. The 452-bp fragment was purified and its extremities were filled in with DNA polymerase. Then the fragment was mixed with pGM301 (*22*) that had been linearized with *Sma*I. The mixture was ligated and we obtained a new plasmid which contained the 452-bp fragment fused with the CaMV ORF III. The new plasmid was completely digested with *Eco*RI. The fragment containing CaMV DNA was purified and its extremities were filled in. This fragment was mixed with pMW701 (*21*) that had previously been linearized with *Bsu*36I and filled in. The mixture was ligated and we obtained plasmid pFS450.

fusing (i) CaMV ORF VII in frame with the end of ORF IV, and (ii) the beginning of ORF V in frame with the CaMV ORF III (Fig. 1). We used this construction because we had antisera raised against the N-terminal extremities of ORF III product (anti-P3 (*20*)) and ORF VII product (anti-P7 (*21*)) and against the C-terminal part of ORF IV product (anti-P4 (*7*)). In the case of a +1 frameshifting event between ORF IV and ORF V two proteins should appear in a Western blot: one protein of 18.5 kDa corresponding to the ORF VII–IV product (P7–P4) and another one of 41 kDa corresponding to the ORFs VII–IV–V–III product-containing fusion protein (see Fig. 1). The 18.5-kDa polypeptide would be detected by anti-P7 and anti-P4 sera whereas the 41-kDa protein would be detected by anti-P7, anti-P4, and anti-P3 sera.

A plasmid construction useful for this approach is pFS450 which was constructed from pMW701 (*21*). pMW701 is a yeast expression vector containing ORF VII (see Fig. 1) under the control of the promoter and 3′ sequence of the yeast ADH1 gene (alcohol dehydroge-

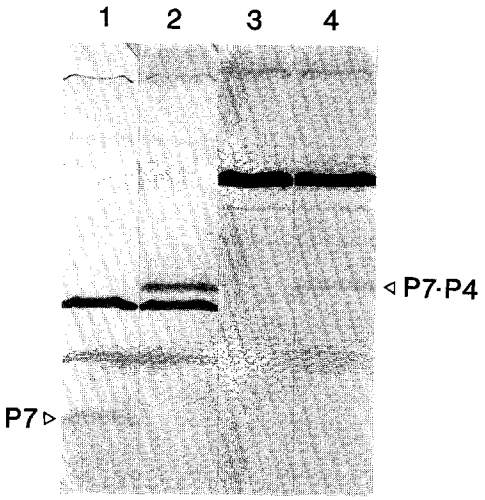

FIG. 2. Immunological detection of P7–P4 in extracts of yeast transformed with pFS450. The proteins were separated on a 0.1% SDS, 15% polyacrylamide gel and electroblotted onto nitrocellulose. The immunoblotting procedure was then performed with anti-P7 serum diluted 1000-fold (slots 1 and 2) or with anti-P4 serum diluted 500-fold (slots 3 and 4). The arrowhead indicates the localization of the proteins detected with anti-P7 and anti-P4. Slots 1 and 3, extracts of *S. cerevisiae* containing pMW701. Slots 2 and 4, extracts of *S. cerevisiae* containing pFS450. Yeast extracts were prepared from 1 ml of cultures grown to an A_{700} 1.80.

nase). We inserted into the *Bsu*36I site of ORF VII located at nucleotide 277 (ORF VII starts at nucleotide 13 and ends at nucleotide 303) the CaMV frameshift sequence fused to ORF III (see Fig. 1) and then introduced pFS450 into *Saccharomyces cerevisiae* strain c13-ABYS86 (pra 1-1, prb 1-1, prc 1-1, cps 1-3, ura Δ3, leu 2-3, 112 his). This strain is particularly suitable because it is deficient in several vacuolar proteinases (*23*).

Yeast cultures containing either pFS450 or pMW701 were grown to an A_{700} of 1.80 and the proteins were extracted as already described (*24*). The polypeptides were separated on a 0.1% SDS, 15% polyacrylamide gel, electroblotted onto a nitrocellulose sheet, and incubated with either anti-P7 or anti-P4 sera. Yeast cells containing pMW701 synthesized a protein of 14 kDa immunodetected by anti-P7 serum (Fig. 2, slot 1) but not by anti-P4 serum (Fig. 2, slot 3). This protein corresponds to the native P7 and has already been described (*21*). Cells containing pFS450 synthesized a protein of 22 kDa immunodetected by anti-P7 and anti-P4 sera (Fig. 2, slots 2 and 4). This is slightly greater than the theoretical molecular weight of P7–P4 (18.5 kDa). It is not surprising since P7 and P4 are known to have apparent molecular weights on SDS–PAGE greater than their theoretical molecular weights (*18, 21*). As our hybrid protein was detected by sera raised against the N-terminal extremity of P7 and the C-terminal part of P4 we can conclude that ORF VII is fused in frame with the end of ORF IV. To verify this, the integrity of the ORF V–ORF III junction was confirmed by DNA sequencing (data not shown).

To look for a +1 frameshifting event we used the anti-P7 serum which detected the protein P7–P4 more efficiently than did the anti-P4 serum (see Fig. 2). Total proteins of yeast containing pFS450 were extracted, separated on a 0.1% SDS, 8–15% gradient polyacrylamide gel, electroblotted onto nitrocellulose and incubated with anti-P7 serum. Two products were immunodetected (Fig. 3, slot 1). One of these is 22 kDa and is the simple translation product of ORF VII–ORF IV (P7–P4; see above). The other, less abundant product is about 50 kDa. This larger species could correspond to either a dimeric form of P7–P4 or a fusion product which can only be produced by a +1 frameshift event.

For this reason we constructed a new plasmid pFS451 which corresponds to pFS450 deleted in CaMV ORF III. The construction of pFS451 is shown in Fig. 4. In the case of a +1 frameshifting event, yeasts containing pFS451 should still be able to synthesize the 22-kDa protein (P7–P4) but not the 50-kDa protein (see Fig. 4). In these new conditions, a 40-kDa protein should be synthesized.

When we compared yeast extracts containing either pFS450 or pFS451, we were unable to detect any differences with anti-P7 serum (Fig. 5), indicating that the

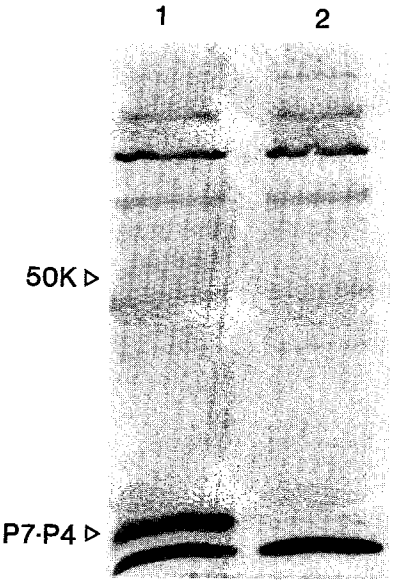

FIG. 3. Immunological detection of a 50-kDa protein in extracts of yeast transformed with pFS450. The proteins were fractionated on a 0.1% SDS, 8–15% gradient polyacrylamide gel. After transfer to nitrocellulose the sheet was incubated with anti-P7 diluted 1000-fold. The arrowheads indicate the localization of the proteins detected with anti-P7. Slot 1, extracts of *S. cerevisiae* containing pFS450. Slot 2, extracts of *S. cerevisiae* containing pMW701.

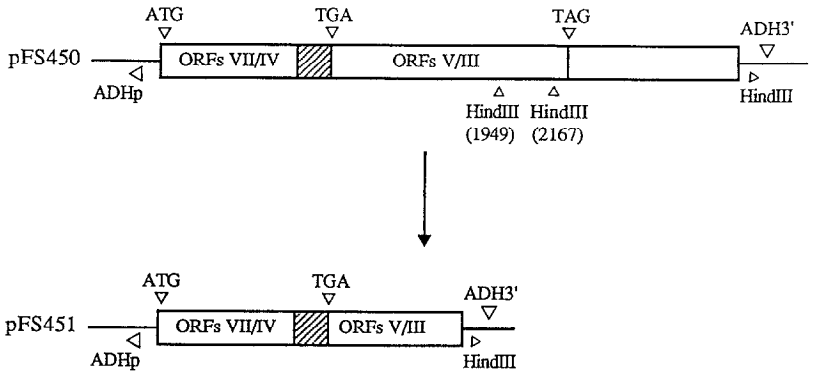

FIG. 4. Construction of plasmid pFS451. Numbers refer to nucleotide positions of *Hin*dIII sites on the CaMV DNA, strain Cabb-S. Boxes represent the CaMV DNA. Thin lines represent the plasmid DNA. The initiation codon of ORF VII, the stop codons of ORF III and ORF IV, the ADH1 promoter, the 3′ sequence of ADH1 gene, and the location of *Hin*dIII sites are indicated by arrowheads. The hatched box represents the CaMV DNA containing the frameshifting region. pFS450 was completely digested with *Hin*dIII. The mixture was ligated and we obtained a deleted plasmid pFS451 which has lost the two-thirds of the ORF III sequence.

product of 50 kDa corresponds probably to aggregates of P7–P4 and is not synthesized by a +1 frameshift event. Yeast proteins were also tested with the anti-P3 serum and no protein was immunodetected (data not shown). The background of the aspecific immunoreactions was reduced by immunoabsorbing all the tested antisera against a mixture of bacteria and yeast cells. Except for the 22- and 50-kDa proteins no other proteins were detected (result not shown). From these results, we conclude that the 50-kDa polypeptide is probably a dimeric form of P7–P4. During the course of these experiments we observed that yeast containing pFS451 expressed more P7–P4 than yeast containing pFS450 (compare slots 1 and 2 of Fig. 5).

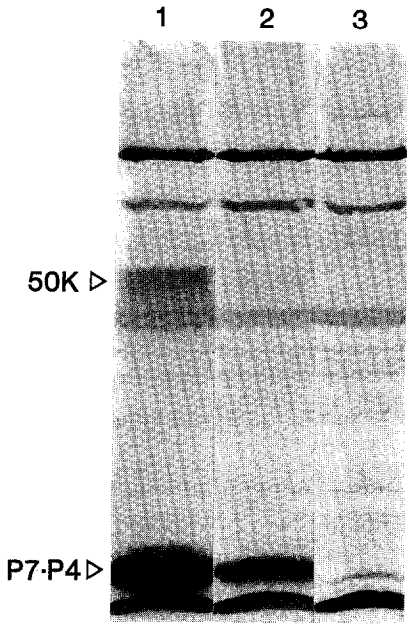

FIG. 5. Immunoblotting with yeast extracts containing pFS451. Yeast extracts were immunoblotted as described in the legend of Fig. 3. Anti-P7 serum was diluted 1000-fold. Slot 1, extracts of *S. cerevisiae* containing pFS451. Slot 2, extracts of *S. cerevisiae* containing pFS450. Slot 3, extracts of *S. cerevisiae* containing pMW701.

In order to address the question of whether the overlapping sequence between CaMV ORF IV and ORF V was able to direct a +1 frameshifting event, we introduced this sequence between CaMV ORF VII and CaMV ORF III. This construct was then expressed in a yeast system known to be able to carry out the frameshifting mechanism. However, no fusion protein synthesized from ORF VII and ORF III could be detected. Three reasons could explain this result. (i) The cloned fragment contained only 452 bp (see results), possibly lacking a potential stem–loop structure positioned downstream of this fragment which might be important for expression. However, in coronaviruses a stretch of 86 nucleotides (*25*) and for Rous sarcoma virus one of 147 nucleotides (*14*) are sufficient for efficient −1 frameshifting. Moreover, for HIV −1 frameshifting (*15*) and Ty +1 frameshifting (*13*) no stem–loop structures downstream from the frameshifting site are necessary. (ii) The absence of a fusion protein could be due to the presence of a transcriptional stop signal located just after the stop codon of ORF IV. However, by Northern blotting we detected full-length RNAs capable of expressing all the proteins from the ORF VII to the ORF III (result not shown). (iii) The CaMV frameshifting event may not be possible in yeast because some necessary plant or viral factors are lacking.

While this work was in progress, Schultze *et al.* (*26*) demonstrated that a CaMV mutant in which ORF IV and ORF V are separated by stop codons in all three reading frames is viable and stable. They proposed that the ORF V is translated separately from the ORF

IV. Our results confirm this hypothesis. Such a mechanism is not unusual and has already been proposed. It has been demonstrated that synthesis of hepadnavirus reverse transcriptase does not require formation of a capsid–polymerase fusion protein (*27, 28*). How the CaMV reverse transcriptase is synthesized is still unknown. It will be of interest to introduce our construction into plant protoplasts to understand how the reverse transcriptase is synthesized.

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