Analysis of Polypurine Tract-associated DNA Plus-strand Priming in Vivo Utilizing a Plant Pararetroviral Vector Carrying Redundant Ectopic Priming Elements*

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Retro-elements (REs),1 the propagation of which involves copying genomic RNA into DNA mediated by reverse transcriptase (RT), are found in organisms representing the major groups of eukaryotes. REs include animal retroviruses, animal and plant pararetroviruses, and retrotransposable elements (1). Although REs are diverse, most share several common features. Important among these is the pol gene product specifying multiple enzyme activities associated with reverse transcriptase (RT) involved in RE DNA synthesis and processing (2, 3).

REs have a different origin of replication for synthesis of each strand of the double-stranded (ds)DNA phase. First, reverse transcription of the genomic RNA into minus-strand DNA is initiated in most REs by a host tRNA (4). During minus-strand synthesis, RT-associated RNase H activity degrades the RNA template (5, 6). This degradation appears to occur in at least two stages. Processive DNA minus-strand synthesis is accompanied by a polymerase-dependent, non-processive RNase H degradation of the template leaving RNA fragments. These fragments are probably further degraded by subsequent polymerase-independent RNase H cleavages (7–9).

The RNA primer for DNA plus-strand synthesis is generated during these steps by RNase H cleavage at a specific site called the polypurine tract (PPT), 13–18 nucleotides long. For REs producing an integrating linear double-stranded DNA with long terminal repeats (LTRs), precision of plus-strand initiation is essential for RE viability as one LTR border is defined by this step. Therefore, understanding the role of the PPT is important, particularly in retroviruses where plus-strand initiation is a possible target for therapeutic control.

Several studies of PPT function have involved analysis of products made by RT added to RNA primer/DNA template combinations in vitro (10–13) but it is not clear how such reactions relate to plus-strand priming in vivo. Mutagenesis of the LTR-associated PPT could render an RE non-functional in vivo limiting usefulness of this approach. Members of the lentivirus and spumavirus groups of retroviruses, and the yeast retrotransposon Ty1, have a second central PPT colinear with the pol gene (14–16), which could be more amenable to analysis in vivo. Studies have shown that mutations in the pol-associated PPT, leaving the colinear pol amino acid sequence unchanged, impaired priming and retroviral replication in transfected cells (17, 18) but did not affect Ty1 transposition in yeast (16). Charneau et al. (18) also inserted a 29-bp linker containing the PPT sequence into a new site in the HIV-1 genome, where it primed DNA plus-strand synthesis. With this approach, the primer could be moved potentially to a new genome site for analysis without interference from colinear functions.

Plant pararetroviruses, such as cauliflower mosaic virus (CaMV), offer great potential to study plus-strand priming because clones of their circular double-stranded DNA genome can be infectious to plants, and, some members of the group have multiple PPT-associated priming sites (19). Different plant pararetroviruses have 1–3 PPTs close to virion DNA discontinuities, which are structurally similar to the HIV-1 pol-associated DNA discontinuity (Fig. 1). CaMV has two PPTs; one is colinear with a non-essential gene and can be deleted without loss of viral infectivity (20, 21). Priming at the CaMV PPTs in vivo is less that 100% efficient (22, 23), but additional priming seems to occur at other PPT-like sequences (24) and by hairpin fold-back (22, 25), although it is not known if these extra events contribute to viral replication. CaMV PPTs have functional polarity (26), and we have recently shown that plus-strand priming can be initiated in vivo by insertion of a PPT at an ectopic genome site (27). These features have enabled us to investigate the pararetroviral plus-strand priming mechanism in vivo.

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‡ The abbreviations used are: RE, retro-element; RT, reverse transcriptase; PPT, polypurine tract; (+)ps, DNA plus-strand priming sequence; bp, base pair(s); kb, kilobase pair(s); PCR, polymerase chain reaction; LTR, long terminal repeat; HIV, human immunodeficiency virus; CaMV, cauliflower mosaic virus.
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EXPERIMENTAL PROCEDURES

Virus, Plants, and Kits—CaMV isolates used were Cabb B-JI and Aust (21), and CM-184 (20) maintained as infectious clones. Infection studies were with turnip plants (Brassica rapa-rapifera, cv. "Just Right"). Celite abrasive was supplied by Celite Corp. pGEM cloning vectors were from Promega. Mutagenesis was performed using the QuikChange procedure (Stratagene). Primer extension was performed with the T7 sequencing kit supplied by Amersham Pharmacia Biotech. Infection of Plants—Turnip seedlings were grown in a greenhouse (minimum day length, 16 h) at 20 °C. Plants were mechanically inoculated using Celite abrasive with sap from infected plants, or cloned viral DNA constructs (1–4 μg/plant) digested with a restriction enzyme to liberate viral DNA from the cloning vector. Symptoms were scored when consistent on a minimum of 4 plants.

Construction of Mutant Viral Genomes—Molecular biological manipulations were based upon those described by Sambrook et al. (28). Artificial plus-strand primers, (+)ps1 elements, were made by annealing pairs of complementary, 5′-phosphorylated oligonucleotides (Table I, oligonucleotides 1–28) generating terminal Nhel sites. The annealed oligonucleotides were cloned into the unique Nhel site introduced into infectious CaMV 35 S promoter deletion mutants M4 and M10 (29) as described by Noad et al. (27). Insert orientation was checked by PCR.

Authentic CaMV (+)ps1 elements were inactivated by mutagenesis as follows. For the (+)ps1 element colinear with gene V, the clone MP12 (27) was digested with Psfl and the fragment (3430–5386) containing the cloning vector (pGEM5) inserted at the unique SalI site (4856), and the site to be mutated (-4210), was self-ligated and cloned. Mutagenesis was performed using the QuikChange procedure with mutagenic oligonucleotides (Table I, oligonucleotides 29 and 30). The mutation was checked by sequencing, and an Nael fragment (pGEM5 site to CaMV site 3910) containing the change was isolated and used to replace the wild-type fragment in the full-length CaMV clone pBJSssl, generating clone PB1. The (+)ps2 priming element colinear with gene II was mutated by exploiting the adjacent unique Xhol site to design an oligonucleotide containing this site and the mutations (Table I, oligonucleotide 31). A fragment was amplified between this and an upstream site (740–761), and an NsiI-Xhol fragment (1042–1644) was isolated from the PCR product. This fragment was used to replace a similar fragment in a BstEI (128)-Xhol subclone of the CaMV genome (producing clone BB5) and this, in turn, replaced the wild-type fragment generating the full-length clone BB8. The (+)ps2 sequence was also deleted using a strategy similar to that for BB8 but employing an oligonucleotide with a 33-bp in-frame deletion (Table I, oligonucleotide 32) relative to the wild-type CaMV sequence, generating clone BB12. Clone BB9, containing both mutant (+)ps1 elements, was made by replacing the BstEI/Xhol-cut fragment from BB8 with that from BB5 producing clone BB9. A mutant genome containing the mutation (+)ps1 and the deleted (+)ps2 was created by replacing the SpeI(110)-Xhol fragment of PB1 with that from BB12 producing clone BB21. Construct PM1 comprised the deleted gene II region of CaMV isolate CM4–184, the mutated (+)ps1 element, and a linker in the 35 S promoter. PM1 was made by ligating the 3.0-kb Stul fragment from clone MP12 (27) to the complementary 7.5-kb Stul fragment of clone PB1. Construct GG5 was a modification of PM1 in which an artificial (+)ps1 element (Table I, oligonucleotides 1–2) was introduced in forward orientation into the Nhel linker in the 35 S promoter. All mutations introduced into the viral genome were checked by sequencing before infection and after recovery from plants if constructs were infectious.

Analysis of Virion DNA Discontinuities—Priming of RNA plus-strand synthesis was determined by detecting discontinuities in CaMV virion DNA purified as described by Covey et al. (30). Denaturing gel electrophoresis of CaMV virion DNA and detection by blot-hybridization was as described by Noad et al. (27). Efficiency of plus-strand priming at the ectopic site (+)ps3 was determined semi-quantitatively by scanning autoradiograms following denaturing Southern blot analysis. Denaturing gel lanes were analyzed using ImageQuant software to integrate peak areas. Precise determination of DNA plus-strand 5′ ends was by primer extension using appropriate primers upstream of the (+)ps2 and (+)ps3 elements, respectively. Primer extension was performed on 1–2 μg of virion DNA using the method described by Mberry et al. (31).

RESULTS

The Polyuridine Tract and 5′-Pyrimidines Are Both Required for Plus-strand Priming in Vivo—We have adopted a new descriptor for plant pararetroviral plus-strand priming sequences, (+)ps, since the term PPT is an incomplete definition and previous abbreviations in pararetroviruses referred to virion DNA gaps rather than priming elements themselves. The two authentic CaMV (+)ps elements are co-linear with viral genes (Fig. 1). The CaMV reverse transcriptase gene (gene V) contains (+)ps1, and the aphid transmission protein gene (gene II) contains (+)ps2. Although gene II is not essential for viral replication and can be deleted, our studies of (+)ps2 have shown that mutagenesis can interfere with co-linear functions affecting CaMV pathogenicity. To circumvent this problem, we exploited our discovery that a redundant 31-bp copy of (+)ps2 inserted into the CaMV 35 S promoter (Figs. 1A and 2A) efficiently primes DNA plus-strands during infection of plants with no detected effect on viral pathogenicity (27). Our assay for functionality of the inserted primer is to measure generation of a new discontinuity in progeny CaMV DNA. Following denaturing gel electrophoresis, virion DNA of the M4 vector, lacking the inserted priming element (Fig. 2A), resolves as three major single-stranded fragments (Fig. 2B) comprising the genome-length product of minus-strand priming (α-strand, 8 kb), and two fragments resulting from plus-strand priming at (+)ps1 (β-strand, 5.4 kb) and (+)ps2 (γ-strand, 2.6 kb). A 3.8-kb fragment (β-strand) is a product of failed second strand transfer during reverse transcription (Fig. 2, B and C). The presence of an additional primer in the 35 S promoter, in vector M4Gf, produces a new discontinuity in the β-strand (Fig. 3). The efficiency of priming at the new site was measured in denaturing gels by assessing the reduction in amount of the 5.4-kb single-stranded DNA fragment and replacement by two additional fragments of 3.1 and 2.3 kb. The amount of residual β-strand DNA in M4Gf shows that priming at the new site is less than 100% effective. By scanning gels, integrating the peak areas (see Fig. 2B), and comparing amounts of β-strand, we

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FIG. 2. Priming in vivo by an ectopic (+)ps3 element. A, in the wild-type (wt) CaMV genome, the 35 S promoter (P35S) and the C-terminal domain of gene VI overlap. An infectious 35 S promoter deletion mutant (M4) contains a unique NheI site into which the (+)ps3 element was inserted in forward orientation producing the infectious CaMV vector M4Gf. B, denaturing gel and blot-hybridization analysis of CaMV virion DNA from plants infected with M4 and M4Gf. Efficiency of priming at (+)ps3 was determined by scanning the lanes, integrating the peak areas (shown in arbitrary units in square brackets) and comparing the relative abundance of the β-strand bands with a band not altered by the ectopic priming event. C, single-stranded DNA components of M4 virion DNA and M4Gf variants. The β-strand in M4 is divided into two components (δ,ε) by the new priming event in M4Gf. The defective (def-) form is produced by a failed second strand transfer.

estimate that the new priming site mediated by oligonucleotide (+)ps3:o (Table I) is recognized in 85–90% of virion DNA molecules.

The relative activity of mutant (+)ps elements was then tested at the ectopic site (Fig. 3). This was quantified by comparing the intensity of the 3.1-kb band (γ-strand) with that of the 2.6-kb band (γ-strand) as described under “Experimental Procedures.” Both of the authentic CaMV (+)ps elements have a similar organization, although the sequences differ slightly. In each case, the PPT is preceded immediately by a pyrimidine-rich sequence then an A-rich region 5' of this (see Fig. 7A). We first tested the involvement of these conserved upstream elements in priming at the ectopic site. Removal of the 7-G nucleotide A-rich sequence (Fig. 4). Removal of 3 of the 7 G nucleotides (construct (+)ps3:x in which the PPT was shortened by 1 nucleotide) reduced priming efficiency to about 68%. A more significant reduction was seen in construct (+)ps3:w and to a lesser extent in (+)ps3:x in which the PPT was shortened by 1 and 2 nucleotides, respectively (Fig. 2). These experiments show that maximal priming activity requires both a PPT sequence of optimal length together with upstream pyrimidines.

Further mutants were constructed to investigate the effects of small deletions and insertions on primer efficiency and primer fidelity (described below). We confirmed involvement of the pyrimidines by removing 2 of the 5 T nucleotides in a construct lacking upstream purines that interfere with priming (Fig. 4, construct (+)ps3:b) and observed a reduction in the level of priming efficiency to 75% of that of the full-length element. In an attempt to increase priming efficiency, we added 3 extra T nucleotides (construct (+)ps3:a), but this produced priming with an efficiency indistinguishable from the wild-type sequence (Fig. 4). Removal of 3 of the 7 G nucleotides (construct (+)ps3:d) significantly reduced priming efficiency to 11%. A slight reduction in efficiency was also found with construct (+)ps3:c containing 2 extra G nucleotides (Fig. 3). These experiments define the limits of the cis-acting elements required for CaMV plus-strand priming in vivo. Thus, maximal priming activity requires 5’ pyrimidines and about 13 nucleotides in the PPT. Loss of more than 3 purines virtually abolishes priming, as does loss of all of the pyrimidines. Increasing the length of the PPT also reduced priming efficiency.

Factors Affecting Plus-strand Priming Fidelity in Vivo—The 5’ ends of the authentic CaMV plus-strands have been mapped previously to fixed positions (32), as is the case with other REs. Therefore, we wanted to determine (i) whether priming fidelity was maintained at the ectopic site and (ii) the effects of mutations on priming precision in vivo. We first checked the plus-
strand 5’ end at the authentic (+)ps2 site in gene II, by primer extension. The 5’ end was found to be at a G residue 3 nucleotides upstream of the 3’ end of the PPT portion of the (+)ps2 site (Fig. 5A). This is 1 nucleotide upstream of that previously reported by Richards et al. (32). In addition to the predominant 5’ end, lesser secondary ends were observed 1 or 2 nucleotides to the 5’ side of the major end. Interestingly, a gene II variant (d2) we have obtained following mutagenesis, with 2 of the PPT G nucleotides substituted for T nucleotides, showed essentially the same priming fidelity as the wild-type sequence (Fig. 5, A and D).

We then determined priming fidelity at the (+)ps3 ectopic site and compared it with that at the authentic site. Priming by (+)ps3:o gave the same predominant 5’ end as was found at the wild-type (+)ps2 priming site (Fig. 5, compare B with A). In different primer extension experiments, the degree of heterogeneity around the major 5’ end sometimes varied. The distribution of ends shown in Fig. 5D has been assessed from two or more primer extension experiments. None of the mutations

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**Table I**

| No. | Mutant Oligonucleotide (5’ → 3’) |
|-----|---------------------------------|
| 1.  | (+)ps3:o CTAGCAAAAACCATTTT TAGAGTGGGGGGTTG |
| 2.  | (+)ps3:p CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |
| 3.  | (+)ps3:q CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |
| 4.  | (+)ps3:r CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |
| 5.  | (+)ps3:s CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |
| 6.  | (+)ps3:t CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |
| 7.  | (+)ps3:u CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |
| 8.  | (+)ps3:v CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |
| 9.  | (+)ps3:w CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |
| 10. | (+)ps3:x CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |
| 11. | (+)ps3:a CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |
| 12. | (+)ps3:b CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |
| 13. | (+)ps3:c CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |
| 14. | (+)ps3:d CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |
| 15. | (+)ps3:e CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |
| 16. | (+)ps3:f CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |
| 17. | (+)ps3:g CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |
| 18. | (+)ps3:h CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |
| 19. | (+)ps3:i CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |
| 20. | (+)ps3:j CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |
| 21. | (+)ps3:k CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |
| 22. | (+)ps3:l CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |
| 23. | (+)ps3:m CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |
| 24. | (+)ps3:n CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |
| 25. | (+)ps3:o CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |
| 26. | (+)ps3:p CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |
| 27. | (+)ps3:q CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |
| 28. | (+)ps3:r CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |
| 29. | (+)ps3:s CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |
| 30. | (+)ps3:t CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |
| 31. | (+)ps3:u CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |
| 32. | (+)ps3:v CTAGCAACCCCGCCACCTTAAAATGGTGGGTTG |

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**Fig. 5.** Analysis of the DNA plus-strand 5’ ends in authentic and ectopic priming sites by primer extension. A–C, primer extension of CaMV wild-type and mutant d2 virion DNA at the authentic (+)ps2 discontinuity in gene II (A) or variants at the ectopic (+)ps3 site (B and C), as described in Figs. 3 and 4, with the respective C-track sequences as markers (M). D, the positions of the DNA 5’ ends defined by selected (+)ps elements determined by primer extension. The size of the arrow reflects the frequency of priming at a particular nucleotide determined from at least two primer extension experiments.
studied altered the position of the major 5' end relative to the 3' end of the PPT. However, the relative abundance and positions of the secondary 5' ends varied in some mutants. For instance, (+ps3 t) showed a slight bias of minor ends to the 5' side and (+ps3 x) to the 3' side of the major 5' end (Fig. 5, B and D). Mutations affecting the 5' pyrimidines had no significant effect on the major 5' end. The most significant effect on plus-strand priming fidelity was observed in construct (+ps3 c) in which 2 additional G residues had been inserted into the PPT. This construct produced two major 5' ends with the predominant end located 3 nucleotides from the PPT 3' end, as before. However, the second major end was displaced 2 nucleotides to the 5' side, which places this end at the same relative position from the 5' end of the PPT as in the wild-type sequence (Fig. 5D). These experiments show that DNA plus-strand 5' end positioning by the ectopic priming element is essentially the same as at the authentic site. Additionally, the start site appears to be controlled by the length of the PPT portion of the (+ps4) element with a primary fixing point at the PPT 3' end.

Priming from Tandemly Arranged (+ps3) Elements—During assembly of one (+ps3) construct into CaMV vector M10, we isolated a clone with three tandem (+ps3:0) elements, each in forward orientation (Fig. 6A). This construct was infectious and relatively stable, although a recombinant with a single (+ps3:0) element was cloned from PCR reactions on progeny virion DNA. Primer extension of the (+ps3:3o) virions showed a correctly positioned DNA 5' end at each of the three priming sites (Fig. 6B). However, the discontinuities resulting from priming at the upstream elements would only be detectable by primer extension if priming at the respective downstream elements had not occurred (Fig. 6C, i–iii). Priming at all three tandem sites in any one virion DNA molecule (Fig. 6C, iv) would not be detectable by primer extension, so we cannot exclude more than one priming event per molecule. An additional 5' end was mapped to a non-authentic purine-rich sequence in the most distal element (Fig. 6B) that we had not seen in other primer extension experiments. This experiment indicates that the presence of adjacent primers does not affect primer fidelity, although there may have been some competition for selection and generation of additional ends due to the complexity of the sequence environment.

Pararetrovirus Infectivity Rescued by an Ectopic Plus-strand Primer—The experiments above demonstrated the relative efficiency of variant (+ps) elements when they were redundant to viral replication. Although primer redundancy is a normal feature of CaMV, we wanted to be sure that priming at the ectopic site could be fully functional in replication. To achieve this, we resolved to destroy authentic CaMV plus-strand priming and to rescue infectivity with a single ectopic priming element. The CaMV (+ps1) element is colinear with the RT gene; therefore, destruction of this primer must not disrupt RT. We introduced single nucleotide changes converting purines to pyrimidines and, vice versa, without changing the RT amino acid sequence, producing construct PB1, which was infectious (Fig. 7, A and B). Progeny PB1 virion DNA isolated from plants had only one discontinuity in each strand producing predominantly genome-length single-stranded DNA fragments (8 kb) on denaturation together with a predicted fragment of 6.4 kb arising from defective second strand transfer (Fig. 7, C and D). Next, the (+ps2) element was disrupted with third nucleotide changes, in construction BB8. This was also infectious with characteristics suggesting priming from the sum of only one (+ps) site (Fig. 7, C and D). To test the effectiveness of both sets of mutations, we produced construct BB9, which should have been completely disabled in plus-strand priming. In the first infectivity test, we inoculated 15 turnip seedlings with BB9 DNA and none showed symptoms. In a second round of BB9 inoculations, 1 plant from 15 showed symptoms. Analysis of the virion DNA from this plant indicated that priming had occurred only at the mutated
Priming of DNA plus-strand synthesis is a critical step in RE propagation. We report here the first detailed study of the cis-acting sequence requirements for this process during RE viral infection of a whole organism. This was facilitated by the ability to manipulate a redundant ectopic (+)ps element in infectious clones of the plant pararetrovirus CaMV. In particular, we were able to study priming, independent of any requirement in replication or pathogenicity. By measuring the percentage of virion DNA molecules containing a new discontinuity at the ectopic (+)ps element, we estimated the approximate efficiency of priming at this site as about 85–90%. We have previously shown that priming at the authentic CaMV plus-strand primers is less than 100% efficient, generating a proportion of progeny DNA molecules lacking a discontinuity at each of the two sites, respectively (23). Therefore, we would not expect priming at the new site to be more efficient than at the authentic primers so 85–90% probably approximates the efficiency of the authentic primers as well.

The shortest artificial primer that gave maximal activity (construct p) was 19 nucleotides long. We conclude that the complete element probably encompasses 18 nucleotides assuming that the 5′-most A residue in this construct is not a part of the primer. Further mutational analysis should confirm this. Deleted variants of the artificial (+)ps3 element which reduced the size of the 13 nucleotide PPT to 10 or fewer purines significantly reduced the efficiency of priming. Priming was still detectable when only the 7 G-box residues of the 13 nucleotide PPT were retained, but deletion of the 7 G nucleotides leaving 5 purines ablated priming. Priming efficiency was also diminished when the length of the PPT was increased by 3 or more nucleotides. This suggests a simple relationship between PPT length and priming efficiency, but other factors must also be important because deletion of the first PPT purine caused a greater loss of priming efficiency than loss of the first 2 purines (Fig. 3).

Unexpectedly, deletion of the 5 pyrimidines (T nucleotides) located immediately 5′ of the PPT (construct q) abolished detectable priming (Fig. 3). Loss of the T nucleotides caused juxtaposition of an upstream stretch of A nucleotides next to the PPT increasing the PPT length from 13 to 20 nucleotides. This change was most likely inhibitory to priming, supporting our conclusion that the PPT has both a minimal and maximal length for optimum efficiency. However, other mutants showed a clear involvement of the 5′ pyrimidines in primer recognition. Although pyrimidine-rich sequences 5′ to the PPT are highly conserved among REs, in vitro experiments with retroviral PPTs have suggested that the 5′ pyrimidines are not necessary for priming (see e.g., Refs. 11 and 12). The differences between CaMV and retroviruses could reflect a fundamental divergence in the priming mechanism. However, due to the conservation of the 5′ pyrimidines among REs, we favor the explanation that some priming reactions in vitro do not accurately reflect all of the priming steps in vivo, as discussed by Klarmann et al. (13). One explanation for this difference might reside in the design of primer/template combinations used in in vitro studies. For example, use of a continuous RNA primer annealed to a DNA template (12) allows assay of only the polymerase-independent RNase H cleavages and excludes the initial round of polymerase-dependent RNase H cleavages generating RNA fragments during reverse transcription (6). Thus, we could rationalize our observation that the upstream pyrimidines play an enhancing role in primer recognition by proposing that they function in vivo during the polymerase-dependent phase.

From our observations, we can propose a hypothetical model for plus-strand priming in CaMV (Fig. 8). We suggest that, indices 3 and 9.4

DISCUSSION

Because of the residual infectivity exhibited by BB9, we made two further constructs in which the (+)ps2 element was deleted. BB21 had a 33-bp in-frame deletion removing (+)ps2, which, together with the (+)ps1 mutations, completely abolished infectivity. We also constructed a hybrid CaMV, PM1, comprising the gene II region from CaMV isolate CM4–184 with its 421-bp deletion removing (+)ps2, the inactivated (+)ps1 element, and a linker in the 35 S promoter capable of receiving a new (+)ps3 element. PM1 was not infectious but infectivity was rescued by insertion of a (+)ps3 element (Table I) into the linker generating construct GG5 (Fig. 7). This clearly demonstrates that CaMV infectivity can be made dependent upon an artificial plus-strand primer in an ectopic genomic location and validates the functional assay of mutants at the redundant ectopic site.

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Fig. 7. Destruction of authentic plus-strand primers and rescue of infectivity with an ectopic primer. A, sequences of wild-type and mutant (superscript m) primer sequences (+)ps1 and (+)ps2 in which third nucleotide changes did not alter the colinear amino acid sequences. The putative priming elements are boxed together with the mutated nucleotides (lowercase letters). The sequence deleted in construct BB21 is shown (→)B. Structure of variant CaMV genomic RNA reverse transcription templates and their infectivity (inf.). Fully functional and destroyed primers are shown as black or open-crossed circles, respectively. C, denaturing gel electrophoresis of virion DNA of infectious constructs BB21, PM1, and GG5 recovered from infected plants. D, structures of variants including the defective strand transfer form of PB1 (def.).
Fidelity of priming at the artificial (+)ps3 site was the same as at the authentic (+)ps2 site, although this was 1 nucleotide different from that previously reported by Richards et al. (32). Since the 5′ pyrimidines had no apparent effect on priming accuracy, processing of the remnants of the pregenome template RNA to give the precise cleavage for priming must only involve interaction between the ribo-PPT and the active site of RNase H on a DNA template (Fig. 8A, iv–vii). Our results are consistent with a model (Fig. 8B) in which there is an apparent preference for interaction between the 3′ portion of the PPT and RNase H since various deletions of the PPT did not alter the position of the primary cleavage site 3 nucleotides upstream of the PPT 3′ end (Fig. 4D). Increasing the length of the PPT by 2 nucleotides again produced the major priming site 3 nucleotides upstream from the PPT 3′ end. However, the presence of a major secondary site 2 nucleotides upstream of this suggests alternative PPT length measurement from the PPT 5′ end (Fig. 8B, ii and iii). This might also explain heterogeneity in priming. An alternative model for primer recognition would involve changes in shape of the RNase H active site to fit around primers of different lengths rather than variations in the way the PPT fits into a fixed-shape active site (Fig. 8B, iv).

It is likely that other structural features are important in defining the 5′ end, as suggested for HIV-1 by Powell and Levin (12). Various studies have shown that purine for pyrimidine substitutions in the PPT affect priming fidelity (11, 16, 18, 33). This remains to be determined for CaMV (+)ps elements, but, in two of our primer mutants in which 2 adjacent PPT G residues were substituted by 2 A and 2 T nucleotides, respectively, no change in the predominant DNA 5′ end was observed. The presence of immediately adjacent, tandem (+)ps elements in one of our constructs also showed no effect of primer proximity on priming fidelity, although there appeared to be competition for primer selection and possibly a novel 5′ end generated in a purine-rich region in the last tandem primer. The precision of the plus-strand 5′ ends in pararetroviruses is intriguing since they do not integrate an LTR-containing provirus with precise boundaries like retroviruses and retrotransposons. This conserved precision suggests that the RT-associated enzyme activities responsible for generating plus-strand primers are inextricably linked to production of a precise start site rather than a frayed end, suggesting that pararetroviral RTs evolved from retroviral or retrotransposon RTs rather than the converse.

We also confirmed that the artificial (+)ps element functioned accurately and efficiently independent of its sequence environment. It also seems to function equally well in at least three other randomly chosen insertion sites (data not shown). Full functionality was confirmed by use of the ectopic primer to rescue infectivity following mutational inactivation of the authentic (+)ps sites. Suppression or removal of the two authentic CaMV (+)ps elements resulted in complete abolition of viral infectivity, suggesting that any minor additional priming sites, previously reported by Maule and Thomas (24), were not sufficient to establish infectivity. These observations raise the question as to the requirement for redundant plus-strand primers in CaMV and other REs. It has been suggested that an additional (+)ps site increases the rate of replication of HIV-1, although it may also be involved in a novel replicative strategy (35). There is no evidence that multiple (+)ps sites in plant pararetroviruses confer a replicative advantage. Pathogenically, CaMV variants with one (+)ps are only marginally less severe than those with two, although this difference could be due to an effect on colinear elements. One explanation for
plus-strand primer redundancy in plant pararetroviruses might lie in their inherent inefficiency. Two or more primers could confer a slight adaptive advantage over a single primer in marginal environments. Even so, the ability to manipulate pararetroviral plus-strand priming mechanisms in vivo opens up new possibilities to understand the relationship between in vitro and in vivo studies of RT and to engineer new types of RT and RE tools.

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