Interplay of two uridylyte-specific RNA binding sites in the translocation of poly(A) polymerase from vaccinia virus

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The VP55 (catalytic) subunit of vaccinia virus heterodimeric poly(A) polymerase (PAP) contacts 31–40 nucleotide segments of RNA in a uridylyte-dependent manner, and effects the rapid, processive addition of a 30 nt oligo(A) tail. Here, the minimum size of uridylyte-containing RNA required for stable VP55 interaction was refined to 33–34 nt. VP55 binding experiments using a set of sixteen 34 nt DNA–RNA chimeras, each containing a differently positioned tetra-uridylyte cluster within an oligo(dC) background, indicated that the protein contacts uridylytes at two positions within the oligonucleotide. Combination of two optimally positioned tetra-uridylyte clusters into a single oligonucleotide fully restored the properties of an optimal substrate, rU₃₄, in VP55 binding and salt-resistant polyadenylation. The positions of the two uridylyte interaction sites, ~10 and ~25 nt from the oligonucleotide 3' OH, were confirmed using a selection scheme employing dC–rU oligonucleotide chimera pools. These and additional data suggest a mechanism for polymerase translocation with respect to RNA comparable with inchworming models of transcriptional elongation. In selection experiments incorporating the PAP-associated processivity factor VP39, the latter was shown to replace the 3' OH-distal uridylyte contact site with one ~10 nt further upstream.

Keywords: poly(A)/polymerase/RNA/selection/translocation

Introduction

Despite steady advances (Wahle and Keller, 1992), mechanisms of poly(A) tail formation are not fully understood. The enzyme responsible for the polymerization of adenylates, poly(A) polymerase (PAP), has been isolated from a variety of organisms including *Escherichia coli*, vaccinia virus and various eukaryotes (Moss *et al.*, 1975; Cao and Sarkar, 1992; Ballantyne *et al.*, 1995; Keller, 1995; Manley, 1995; Martin and Keller, 1996; Ohnacker *et al.*, 1996). Studies of the vaccinia enzyme have shown it to be a heterodimer (Moss *et al.*, 1975), the genes for whose subunits (referred to as VP55 and VP39) have been identified (Gershon *et al.*, 1991). The VP55 subunit possesses full PAP catalytic activity (Gershon *et al.*, 1991). Purified, recombinant VP55 has the unique property, *in vitro*, of polyadenylating mRNA 3'-end-related primers bimodally: 30–35 adenylates are added in a rapid,

highly processive initiating burst, followed by an abrupt transition to a slow, non-processive mode of adenylate addition. By contrast, oligo(A) primers are polyadenylated only in the slow non-processive mode. By polyadenylation of variants of the mRNA 3'-end primer containing pre-formed 3' oligo(A) extensions, the abrupt transition from processive to non-processive polyadenylation was shown to be regulated by the net length of the oligo(A) tail rather than by the number of adenylate additions catalyzed by VP55 (Gershon and Moss, 1992). Although the VP39 subunit of the vaccinia PAP possesses no polyadenylation catalytic activity (Gershon *et al.*, 1991), it dramatically accelerates the VP55-catalyzed extension of either mRNA 3'-end primers with short oligo(A) tails, or poly(A) primers (Gershon and Moss, 1993a). This acceleration is effected through a conversion of the VP55-catalyzed slow, non-processive polyadenylation that occurs after the initial burst, back to a much more processive reaction. This action indicates that VP39 somehow anchors VP55 to the growing poly(A) tail.

The initial polyadenylation burst by monomeric VP55 was shown to be independent of specific RNA sequences (Gershon and Moss, 1993b), though the presence of uridylyte residues within the 3' 31–40 nucleotides of the RNA primer was crucial. The precise positioning of the uridylytes did not appear to be critical provided the overall uridylyte content was greater than ~33%. The uridylyte specificity of VP55 was underscored by the finding that even DNA, in which the deoxythymidylate residues were replaced with ribouridylytes, could be polyadenylated efficiently by VP55 with a processive burst (Gershon and Moss, 1993b). Evidence was also presented that VP55 translocates as it processively adds the 30 nt oligo(A) tail, and that it is not the length of oligo(A) *per se* which is measured by VP55, but rather the length of uridylyte-free RNA at the 3' terminus (Gershon and Moss, 1993b). A simple model for the translocation of VP55 was introduced, based upon the above data (Figure 9 of Gershon and Moss, 1993b). In the current study, the numbers and arrangements of uridylytes required for an initiating burst of poly(A) tail formation by VP55 have been characterized further. The data indicate the possession of dual uridylyte-specific RNA binding sites by VP55, and indicate the occurrence of RNA–protein transactions comparable with those which may occur during transcriptional elongation by DNA-dependent RNA polymerases (Chamberlin, 1995).

Results

Minimum RNA length required for stable interaction with VP55

It was shown previously that VP55 can form stable complexes with RNA segments 31–40 nt in length, by using the electrophoretic mobility shift assay (EMSA) in

combination with a 3'-co-terminal nested set of RNA oligonucleotides corresponding to the segment of a vaccinia growth factor gene transcript immediately preceding the poly(A) tail ('VGFmer' RNAs, Gershon and Moss, 1993b). Here, the minimum length of RNA required for stable interaction with VP55 was defined further by synthesizing and testing a nested set of VGFmer RNAs 32, 34, 36 and 38 nt in length (Figure 1A). As controls, the previously used 30 and 40 nt RNAs were also included. Whereas only very faint complexes were observed for the 30 and 32mer RNAs, dramatically more abundant complexes were observed for RNAs 34 nt in length and greater (Figure 1B). Maximum complex abundance appeared to correlate with RNAs 36 nt in length or greater (Figure 1B). We conclude that increasing the RNA length to 33 or 34 nt or greater correlates with large increases in binding stability. The modest increases in stability with further length increases beyond 34 nt could have resulted from either the additional length or the increased choice of binding register.

Effect of position of a tetra-uridyate patch within a 34 nt oligonucleotide

The interaction of VP55 with 31–40 nt RNA segments was shown previously to be uridyate dependent (Gershon and Moss, 1993b). Even DNA, in which deoxythymidylates were replaced with ribouridylates, could interact stably with VP55 and become efficiently polyadenylylated. However, the numbers and arrangements of uridylates required for stable interaction and polyadenylation were not clearly ascertained. We set out to investigate their optimal positioning using a set of 16 oligonucleotides in which a cluster of four adjacent uridylates ('tetraU patch') was placed at various positions within minimum binding length (34 nt) oligonucleotides. The oligonucleotides were otherwise composed of oligo(dC), excepting the sugar of the extreme 3' nucleotide, which was always ribose. Cytidine was chosen for background sequences since oligo(C) is highly refractory to binding and polyadenylation by VP55 (Gershon and Moss, 1993b; our unpublished data). The position of the tetraU patch within the 16 'tetraU-scanmer' oligonucleotides was moved from one end to the other, in dinucleotide increments (Figure 2A). Although each of the 16 oligonucleotides was a DNA–RNA chimera, equivalent binding and polyadenylation results were obtained upon assaying a sample of four tetraU-scanmer oligonucleotides re-synthesized entirely from ribonucleotide monomers (data not shown).

Each of the 16 tetraU-scanmers was 5'-end labeled and tested for stable interaction with VP55 in the EMSA. To mimic polyadenylation conditions as closely as possible without actually polyadenylating the oligonucleotides, unlabeled magnesium-cordycepin triphosphate (Mg-CoTP) was included in EMSA incubations. Oligonucleotides could be extended by a maximum of only a single nucleotide under these conditions. For each of the oligonucleotides, complexes with VP55 were much less abundant than that observed using oligo(U) of the same length (Figure 2B). However, the weak complexes which were observed varied in abundance from one oligonucleotide to another. Thus, nodes of EMSA complex abundance correlated with tetraU positions centered ~10 and ~24–26 nt from the 3' OH. Upon repeating the

Fig. 1. (A) Sequences (5' to 3') of the 3'-co-terminal nested set of 'VGFmer' RNAs 30–40 nt in length (see text) used for EMSA experiments. Distances (nt) from the 3' OH are indicated. (B) EMSAs for the VGFmer RNAs shown in (A), in the absence and presence (– and + respectively), of VP55. 'C' = VP55–RNA complex, 'F' = free RNA.

experiment in the absence of Mg-CoTP, a similar result was obtained except that the bifurcation of the binding peak was less pronounced (Figure 2C). In replicate experiments, levels of bifurcation were variable in both the presence and absence of Mg-CoTP (data not shown).

Polyadenylation of each of the 16 tetraU-scanmers was assayed in the presence of 10, 60 and 150 mM NaCl. In contrast to the rU₃₄ control RNA, none of the 16 could be polyadenylated in the presence of 150 mM NaCl (data not shown). This salt sensitivity of the 16 oligonucleotides in polyadenylation correlated with their inability to form abundant complexes with VP55 in the EMSA. At 60 mM NaCl, modest polyadenylation of some of the oligonucleotides was observed (data not shown). The most efficient polyadenylation was observed at 10 mM NaCl (Figure 3A). For the majority of the oligonucleotides, a rapid processive burst of polyadenylation was followed by slow elongation of the oligo(A) tail, which is typical of the action of VP55 (Gershon and Moss, 1992, 1993b). Although patterns and levels of polyadenylation were highly variable between each of the 16 oligonucleotides (Figure 3A), the following trends could be discerned: (i) moving the center of the

Fig. 2. (A) Sequences (5' to 3') of the 16 'tetraU-scanmer' oligonucleotides. The extreme 3'-terminal nucleotide of each oligonucleotide contained a ribose sugar. With this exception, all of the C nucleotides contained deoxyribose sugars. After synthesis and purification, the identities of the oligonucleotides were confirmed by 5'-end labeling followed by complete acid hydrolysis or partial alkaline hydrolysis (data not shown). (B) EMSA of the 16 tetraU-scanmer oligonucleotides whose sequences were given in (A), in the presence of 1 mM Mg-CoTP. Numbers above each lane identify the individual oligonucleotides. The extreme 3'-terminal nucleotide of the oligonucleotide labeled dC₃₄ possessed a ribose sugar [i.e. it was (dC)₃₃rC]. 'C' = VP55-RNA complex, 'F' = free oligonucleotide. Although each oligonucleotide was also electrophoresed in the absence of VP55, these lanes are not shown. The faint band present in all lanes, migrating between the free oligonucleotide and the complex, was also observed upon electrophoresis of the free oligonucleotides. (C) As for (B), except that CoTP was not included in the experiment.

tetraU patch away from the oligonucleotide 5'-end to position -16 with respect to the 3' OH (i.e. from oligonucleotides 1 to 9), in increments of 2 nt, correlated with progressive increases in the lengths of tails added in the processive burst (first timepoint) from ~6–7 nt to ~35 nt; (ii) polyadenylation was not observed for oligonucleotides with tetraU patches centered 12–14 nt from the 3'

OH (i.e. for oligonucleotides 10 and 11); (iii) for tetraU patches centered 0–10 nt from the 3' OH, two sets of polyadenylation products were observed: the first set comprised short ('mini-burst') products whose average length increased by ~2 nt for each 2 nt incremental movement of the tetraU patch, from ~3–4 nt nodal length (oligonucleotide #12) to ~11–12 nt (oligonucleotide #16).

Fig. 3. (A) Polyadenylation of the 16 tetraU-scanmer oligonucleotides and two control oligonucleotides in the presence of 10 mM NaCl. The oligonucleotide labeled dC₃₄ is actually (dC)₃₃rC (as in Figure 2B). The four lanes for each oligonucleotide represent (left to right) 0, 15, 40 and 120 s timepoints from the polyadenylation assay. 'Proximal (EMSA)' and 'distal (EMSA)' refer to the two nodes of the bifurcated binding peak observed by EMSA in the presence of Mg-CoTP (Figure 2B). 'M' (to the right side of the gel) points to the 'mini-burst' polyadenylation products for oligonucleotides 12–16. Migration rates for single-stranded DNA markers are given to the right. (B) Polyadenylation of tetraU-scanmer oligonucleotide 13 at various oligonucleotide:VP55 molar ratios, established by making serial 3-fold dilutions of the oligonucleotide. 9, 3 and 1 represent relative oligonucleotide:VP55 molar ratios. Timepoints were taken as in (A). Migration rates for single-stranded DNA markers are given to the right.

The second set (the 'main burst' products) was always ~35 nt longer than the mini-burst products. Although this characteristic pattern was very faint for oligonucleotide #15, it was qualitatively comparable. These data are interpreted below (see Discussion).

To determine whether both sets of polyadenylation products for oligonucleotides 12–16 were generated processively, a representative oligonucleotide from this group (#13) was polyadenylated at various ratios of oligonucleotide to VP55 (Figure 3B). We deduce that both sets are produced processively, since the abundance of products changed, but their rates of formation remained constant. It was not clear from this experiment whether the mini-burst products are extended for a few nucleotides in a non-processive manner, prior to initiation of the main polyadenylation burst.

VP55–RNA contact analysis by single-round ligand selection

Assuming a VP55 preparation with homogeneous RNA binding characteristics (see below), the above data indicated the possession by VP55 of multiple (two?) oligonucleotide contact sites. These might show selectivity for either the ribo sugar, the uridylylate base or both. To address this further, we devised a single-round selection scheme employing oligonucleotide chimera pools that comprised deoxyriboC homopolymers spiked randomly with riboU. RiboU spiking levels of 10% were employed, since this level had been determined, empirically, to be adequate for

the selection schemes employed (data not shown). The selection step comprised either: (i) an EMSA using 5'- or 3'-end-labeled oligonucleotide, followed by excision of the VP55–oligonucleotide complex from the non-denaturing gel and extraction of the selected oligonucleotide species; or (ii) incubation of the unlabeled oligonucleotide pool with a ³²P-labeled chain-terminating nucleotide analog (CoTP, Thomson and Gershon, 1995) and either VP55 or VP55 + VP39, to effect the selective 3'-end labeling of ranking pool members. The selectivity of the PAP for CoMP transfer would be expected to reflect its substrate specificity for addition of the first nucleotide of the poly(A) tail. Pool members selected by either method were partially hydrolyzed with hot alkali, and the hydrolysis products were electrophoresed in a denaturing gel. The overall levels of hydrolysis employed (~20–30%) were expected to provide no more than a single 'hit' per oligonucleotide molecule. A control experiment (Figure 4A) confirmed that all positions within a non-selectable RNA oligonucleotide, rU₃₄, were hydrolyzed at close to equivalent rates after 5'-end labeling. Relatively uniform hydrolysis patterns were also observed with the oligonucleotide pools to be employed in selection experiments (below) after 5'-end labeling (i.e. in the absence of selection, Figure 5 'free' lanes and data not shown). However, after 3'-end labeling and hydrolysis of rU₃₄, a strong node was observed migrating with the 8 nt hydrolysis product, accompanied by hypo-hydrolysis of ~1–8 nt fragments (Figure 4A). This effect was also observed with the oligonucleotide

Fig. 4. (A) Hot alkaline hydrolysis of the RNA oligonucleotide U_{34} after either 5'- or 3'-end labeling. For each labeling reaction, the three lanes represent (left to right) unhydrolyzed oligonucleotide, 10 min and 20 min hydrolysis. 3'-End labeling followed by hot alkali treatment always led to a strong band migrating with the 8 nt hydrolysis product, accompanied by hypo-hydrolysis of ~1 to ~8 nt oligonucleotide fragments. We also observed this unusual effect after hydrolysis of U_{34} that had been gel purified after 3'-end labeling, after RNase A as well as alkaline treatment of U_{34} , and also following use of yeast PAP as the 3'-end labeling enzyme (data not shown). It could therefore be attributed to neither non-RNA products in the labeling reaction, high pH *per se*, nor an activity specific to the vaccinia enzyme. It could not be recapitulated by incubating 5'-end-labeled RNA with all of the 3'-end labeling reagents except 3'-end labeling enzyme, indicating that the RNA was not being modified directly by these reagents (data not shown). It was not observed when 5'-end-labeled, chemically synthesized U_{34} -3'dA was hydrolyzed, indicating that it could not be attributed to the presence of the 3' dA residue *per se*. It was RNA sequence independent (data not shown), but more noticeable with RNA than with RNA-DNA chimeras (B and C), indicating a requirement for backbone ribose sugars. Thus, by process of elimination, it was apparently attributable to the presence of ^{32}P -labeled CoMP nucleotide attached to the RNA 3'-end. Perhaps the ^{32}P CoMP moiety leads to an RNA modification involving a ribose at approximately position -8, such as an intramolecular cross-link. (B) Partial alkaline hydrolysates of (dU/rU) oligonucleotide pools 34 and 50 nt in length (each synthesized with a riboU nucleotide at the extreme 3' terminus), after selection by ^{32}P CoMP transfer. CoMP transfer reactions were conducted for 5 min in the presence of VP55 and ^{32}P CoTP, and in the absence (-) or presence (+) of VP39. For each selection experiment, the three lanes represent (left to right) unhydrolyzed oligonucleotide, 10 min and 20 min hydrolysis. The approximate sizes of hydrolysis products, ± 2 nt (i.e. the approximate distances of hydrolysis sites from the 3' OH), are given to the right. (C) Equivalent experiment to that shown in (B) except for the use of (dC/rU) oligonucleotide pools 34 and 50 nt in length (synthesized with a riboC nucleotide at the extreme 3' terminus). 'P' and 'D' refer to the apparent positions of the 3' OH-proximal and -distal uridylylate recognition sites, respectively. The dashed line represents the distance through which the distal contact position shifts in the presence of VP39. (D) 3'-End labeling of the RNA A_{50} and of two oligonucleotide pools by VP55 in the absence (-) or presence (+) of VP39, followed by denaturing gel electrophoresis. The ^{32}P -labeled oligonucleotides are shown. The abundance of labeled (dC/rU) $_{34}$, (dC/rU) $_{50}$ and A_{50} were enhanced in the presence of VP39 by factors of 1.5, 4.5 and 14.5, respectively.

Fig. 5. Partial alkaline hydrolysates of the (dC/rU)₃₄ oligonucleotide pool used in Figure 4C, after selection by EMSA complex formation. The leftmost and center sets of hydrolysates are from starting and complexed 5'-end-labeled (dC/rU)₃₄, respectively. The rightmost set of hydrolysates is from (dC/rU)₃₄ after 3'-end labeling using VP55, followed by EMSA complex formation (i.e. double selection). Numbers to the right of the hydrolysates of the complexed oligonucleotides refer to the distances of hydrolysis sites from the oligonucleotide 3'-end. Positions corresponding to the extreme 5'- and 3'-ends of the oligonucleotide are indicated (5' and 3', respectively). The hydrolysates were electrophoresed in a 20% polyacrylamide gel. The absence of the strong band co-migrating with 8 nt fragments, as was seen after selection by 3'-end labeling alone (Figure 4A–C), indicated that this artifact (legend to Figure 4A) was selected against during EMSA complex isolation.

pools used for selection experiments, though it was less pronounced. Extensive analysis (see legend to Figure 4) indicated that the unusual artifact might be due to an interaction between the ³²P-labeled CoMP attached to the oligonucleotide 3'-end and the ribose at approximately position –8. Hydrolysis patterns for rU₃₄ were not significantly different from those shown in Figure 4A after 3'-end labeling in the presence of VP39 (data not shown). Finally, as a safeguard to maximize the discrimination of selected from unselected species, pilot timecourse experiments were performed for each chimera with both VP55 and VP55 + VP39, to establish reaction times over which oligonucleotides were [³²P]CoMP labeled to only <25% of their maximal specific activities (data not shown).

An initial selection experiment was designed to determine whether VP55 has a strong requirement for ribose at any position within the oligonucleotide substrate. Two chimera pools, (dU/rU)₃₄ and (dU/rU)₅₀ were employed, and the result of the selection experiment is shown in Figure 4B. An over-representation of riboU at any position within the selected material would be expected to lead to over-representation of the corresponding band(s) after

hydrolysis, due to the greater sensitivity of the ribose than the deoxyribose sugar to hydrolytic agents. In the presence of VP55 alone, the two oligonucleotides showed similar levels of hydrolysis at all positions, with the possible exception of position –3, at which enhanced hydrolysis was apparent for both oligonucleotides. Since the method was incapable of probing the extreme 3'-end sugar of the oligonucleotide (the extreme 3'-end nucleotide was synthesized with 100% ribose), selectivity for ribose at this position was unknown. We conclude that, with the possible exception of positions –1 and –3, VP55 does not appear to exhibit a strong preference for ribose over deoxyribose sugars within the RNA substrate for addition of the first nucleotide of the poly(A) tail. Similar results were obtained for the two oligonucleotide pools in the presence of both VP55 and VP39 (Figure 4B), except for the possible occurrence of mildly ribose-selective contacts in the region –25 to –30 for the 34 nt chimera.

To probe for the importance of uridylylate bases at any position, a selection experiment was next performed using the oligonucleotide pools (dC/rU)₃₄ and (dC/rU)₅₀. As in the previous experiment, the selection step comprised [³²P]CoMP transfer. Since VP55 appeared to be largely indifferent to the identity of the sugar (Figure 4B), it was expected that the sugar could act as a reporter to read out the identity of the associated base. Furthermore, since oligo(C) is entirely refractory to stable complex formation with, and polyadenylation by, VP55 (above, and Gershon and Moss, 1993b), enhanced hydrolysis at any position would indicate the selection by VP55 of uridylylates at that position. After selection by [³²P]CoMP transfer in the presence of VP55 followed by partial hydrolysis, a distinctive bi-nodal ladder of products was observed for each of the two dC/rU pools (Figure 4C). The two nodes (3' OH-proximal and -distal) appeared to be centered 1 to ~5 and ~21 to ~26 nt, respectively, from the oligonucleotide 3' OH. These data suggested a requirement for uridylylates in two regions of the RNA substrate for addition of the first nucleotide of the poly(A) tail by VP55. Because of the artifactual band migrating with ~8 nt hydrolysis products, it was not clear whether an additional hydrolysis node might be present in this region of the gel. A hydrolysis node might be expected at this position, since tetraU patches at corresponding positions within tetraU-scanmer oligonucleotides can mediate CoMP addition (data not shown). In the presence of VP39, the distal hydrolysis node showed little or no change for the 34 nt oligonucleotide. However, for the 50 nt oligonucleotide, a dramatic (10 nt upstream) shift was observed in the distal node, so that it was now centered at approximately position –35 instead of approximately –25 (Figure 4C). This experiment strongly indicated that, for addition of the first nucleotide of the poly(A) tail to a 50 nt RNA, VP39 either replaces the VP55 distal contact site with uridylylate-selective contacts positioned further upstream, or alters the RNA binding properties of VP55 so that the latter changes its contact site to a more upstream position.

The observation of VP39-induced oligonucleotide–protein contacts ~30 to ~40 nt from the RNA 3'-end for a 50mer, but no change in pattern for a 34mer, suggested that the enhancement of 3' CoMP transfer by VP39 might be substrate length dependent. Although VP39 has been shown to enhance [³²P]CoMP transfer to RNAs 40 and

50 nt in length (Thomson and Gershon, 1995), its effect on 34 nt oligonucleotides, i.e. the minimum binding site size for VP55, was unknown. Therefore, the two dC/rU chimeras employed for hydrolysis in Figure 4C, along with a control RNA (A_{50}), were 3'-end labeled by VP55 in the presence and absence of VP39 (Figure 4D). As predicted, VP39 exerted a significantly greater effect on the labeling of the 50mers, A_{50} and (dC/rU) $_{50}$, than the 34mer (dC/rU) $_{34}$. These data suggest that the VP39-conferred ribouridylate-selective contacts upstream of the 34 nt VP55 contact region play a significant role in the addition of the first nucleotide of the poly(A) tail.

The above data, indicating dual uridylate recognition sites, were confirmed using alternative RNA labeling and selection methodologies. Thus, 5'-end-labeled oligonucleotide pool molecules were selected by EMSA complex formation, followed by partial hydrolysis of the selected molecules. Since selection was based on VP55 binding rather than nucleotidyl transfer, a minimum sized (34 nt) dC/rU oligonucleotide pool was used in order to maintain, as far as possible, a fixed binding register of oligonucleotide with respect to protein. Whereas the control lane (starting oligonucleotide) showed a fairly uniform partial hydrolysis pattern (Figure 5), the pattern for the complexed oligonucleotide showed a pair of nodes, centered ~10 and ~25 nt from the oligonucleotide 3' OH. An additional, double selection experiment was performed by [32 P]CoMP transfer followed by EMSA complex isolation. The hydrolysis pattern resulting from double selection (Figure 5, '3' label') showed two very strong nodes, providing the most dramatic demonstration of any experiment that VP55 contacts RNA at dual uridylate recognition sites. Again, the nodes were centered ~10 and ~25 nt from the oligonucleotide 3' OH. Some evidence for contacts very close to (1–2 nt from) the 3' OH was also apparent after double selection, presumably due to the importance of these contacts for 3'-end labeling (Figure 4C).

Confirmation of dual uridylate contact sites within individual VP55 molecules

To address the possibility that the observations being interpreted as dual uridylate contact sites instead resulted from the presence of two populations of VP55 within protein preparations, two additional oligonucleotides were synthesized, each containing dual tetraU patches (referred to as 'double-tetraU', Figure 6A). Each was a composite of two of the 'tetraU-scanmer' oligonucleotides (Figure 2A), i.e. numbers 4 + 12 and 4 + 13. Three of the individual tetraU-scanmer oligonucleotides, numbers 4, 12 and 13, were assayed as controls. As additional controls, two 'octaU' oligonucleotides were synthesized, in which the individual tetraU patches of tetraU-scanmer oligonucleotides 4 and 12 were expanded individually to eight residues by replacing the two dC residues on either side of the tetraU patch with two additional uridylates (Figure 6A). Although the resulting two 'octaU' oligonucleotides possessed the same number of uridylates as the 'double-tetraU' oligos, in each case the uridylates were confined to a single patch. Each of the oligonucleotides was subjected to the EMSA and polyadenylation assays (Figure 6B and C). In the EMSA (Figure 6B), VP55-oligonucleotide complexes obtained with the double-tetraU

Fig. 6. (A) Sequences (5' to 3') of oligonucleotides used to confirm the possession by VP55 of dual uridylate recognition sites. (B) EMSAs of double tetraUmers ('double'), with controls comprising tetraU-scanmers ('tetra'), octaUmers ('octa'), the RNA U_{34} and the chimera (dC) $_{33}$ rC (labeled 'dC $_{34}$ '). Their sequences are given in (A) and in Figure 2A. 'F' = free oligonucleotide, 'C' = oligonucleotide-VP55 complex. (C) Polyadenylation assays of the oligonucleotides used in (B). For each assay, the three lanes represent (left to right) timepoints of 0, 40 and 120 s. Assays were conducted in the presence of 150 mM NaCl.

oligonucleotides were comparable in abundance with that with the rU_{34} control, indicating a full restoration of VP55-oligonucleotide affinity when two uridylate patches were present, separated by 12–14 nt of non-uridylate-

containing nucleic acid. By contrast, complexes from the two octaU oligonucleotides were much less abundant, comparable with those of the negative controls (progenitor tetraU-scanmer oligonucleotide 4, 12 and 13). In the polyadenylation assay (Figure 6C), whereas the tetraU and octaU oligonucleotides were refractory to polyadenylation at high salt, the double-tetraU oligonucleotides could be polyadenylated in the same efficient manner as the positive control RNA rU₃₄. However, only a short (~4–5 nt) burst of polyadenylation was observed for the double-tetraU-scanmers, compared with the ~14–15 nt burst observed for rU₃₄ (see Discussion). The above experiments clearly indicated that two short ribouridylate patches, which together comprise <24% of the total number of residues of the oligonucleotide, could largely restore the VP55 binding and polyadenylation efficiencies to those for an RNA composed entirely of oligo(U), one of the most efficacious oligonucleotide substrates available. Furthermore, whereas the previous experiments could be interpreted in terms of two distinct sub-populations of VP55, the efficacy of the double-tetraU oligonucleotides eliminated this possibility in favor of the possession of two uridylate recognition sites per VP55 molecule.

Discussion

We have provided evidence that VP55 [the vaccinia virus poly(A) polymerase] recognizes uridylate residues within two major regions of its RNA substrates, via two uridylate recognition sites (denoted the 3' OH-distal and -proximal). For both EMSA complex formation with minimum length oligonucleotides and nucleotidyl transfer, the 3' OH-distal site appears to interact with RNA regions centered ~25 nt from the 3' OH. For nucleotidyl transfer alone, VP55 appears to also interact with an RNA region centered between 1 and ~5 nt from the 3' OH (Figures 3A and 4C) via a site denoted proximal site B which, from its position, would be associated with the nucleotidyl transferase catalytic site. For EMSA complex formation, 3' OH-proximal interactions appear to require a uridylate-containing RNA region centered ~10 nt from the 3' OH (Figures 2B and C and 5) via an RNA-binding site denoted proximal site A. It is not clear whether proximal site A participates in nucleotidyl transfer, due to the presence of hydrolysis artifacts in selection experiments employing 3' CoMP transfer (Figure 4). The arrangement of these sites is schematicized in Figure 7, part 1. Since the 3' OH-distal uridylate recognition site appears to extend no further than ~28 nt in the 5' direction (Figure 4C), yet the minimum RNA length requirement for the formation of abundant VP55–RNA complexes in the EMSA is 33–34 nt (Figure 1B), an additional, non-uridylate-specific site, interacting with RNA 28–34 nt from its 3' OH is suggested (Figure 7, part 1).

Despite the 33–34 nt minimum RNA length requirement for abundant VP55–RNA complex formation, increasing RNA lengths beyond 34 nt, towards 40 nt, led to additional enhancements in EMSA complex abundance (Figure 1B). This effect may have resulted from either (i) the full RNA binding site of VP55 being a little greater than 34 nt in length, or (ii) RNAs greater in length than the 34 nt minimum having more choices of binding register, particu-

Fig. 7. Schematic showing the arrangement of VP55's RNA-binding sites, and their possible dynamic relationship during addition of oligo(A) tails to the oligonucleotides studied herein. The two large rectangles shown juxtaposed in step 1 represent two domains of VP55 whose relative positions can change (illustrated in parts 2–4 by the extension of a black bar connecting the two domains). The black line beneath VP55 in step 1 represents the terminal 34 nt of an RNA substrate containing uridylate residues ('U'), and continuing in a 5' direction with a dashed line. The speckled bar associated with VP55, labeled 'N', refers to a putative VP55 RNA-binding site that makes base non-specific contacts ~28 to ~34 nt from the RNA 3' OH. The black bars labeled 'D' and 'P' represent the 3' OH-distal and -proximal uridylate-specific RNA contact sites of VP55, respectively, as described in the text. For tetraU-scanmer oligonucleotides 1–9, 'D' is envisaged to interact initially with uridylates within a region of the RNA substrate centered ~25 nt from the 3' OH (step 1), and to remain 'clamped' to this RNA region during tail elongation (clamp represented by a short vertical black line in steps 2–4). 'P' incorporates two sub-sites, denoted 'A' and 'B'. Site A is envisaged to interact with uridylates within a region of the substrate RNA centered ~10 nt from the 3' OH. Site B is envisaged to interact with the substrate RNA between the 3' OH and approximately position ~5, and to incorporate the catalytic site. Tail elongation might be accompanied by a change in the relative positions of the two VP55 domains (shown in parts 2–4) and translocation of the RNA with respect to the proximal sites. At some point, restriction to further relative movement of the domains would prevent further tail elongation. For tetraU-scanmer oligonucleotides 12–16, in which the uridylate patch is too close to the oligonucleotide 3' OH for simultaneous interaction of the patch with the distal site and the 3' OH with the catalytic site (Figures 2A and 3A), it is suggested that the uridylates initially interact with VP55 via the 3' OH-proximal site alone. The two sets of polyadenylation products (Figure 3A) would be generated in a two-step process as described in the text. For VP55 to extend tails to an overall length of ~35 nt (as opposed to the addition of 35 adenylates *per se*), tail elongation might be initiated with the polymerase in a partially extended conformation (step 2 or 3).

larly since VP55 does not strongly target the extreme 3' OH for RNA binding stability (as indicated in previous EMSA experiments, e.g. Figure 2 of Gershon and Moss, 1993b). Further evidence that small increases in oligonucleotide length beyond 34 nt can lead to small enhancements in complex abundance came from experiments in which 34mer oligonucleotides were extended at the 3'-end by only a single adenylate, either during chemical

synthesis or by enzymatic CoMP transfer (L.Deng and P.D.Gershon, unpublished results). However, minimum length (34 nt) oligonucleotides were used in the experiments presented here to minimize the potential for interaction with VP55 in multiple binding registers.

Sixteen 34 nt DNA–RNA chimeras were synthesized, each containing a tetra-uridylylate patch within an oligo(dC) background ('tetraU-scanners'). The extreme 3'-terminal nucleotide always contained a ribose sugar. The use of deoxyC in other positions was justified by the finding that four of the oligonucleotides, when re-synthesized with oligo(rC) backgrounds, were functionally comparable with their oligo(dC) equivalents (L.Deng and P.D.Gershon, unpublished results). Moreover, properties comparable with those of the tetraU-scanners used here were exhibited by four RNA oligonucleotides possessing comparably positioned tetraU patches within mixed background sequences (Gershon and Moss, 1993b). Although no single tetraU-scanner showed the same EMSA complex abundance as the positive control RNA rU₃₄, weak complexes were observed. 'Scanning' of the position of the tetraU patch within the oligonucleotide led to either one or two peaks of binding activity (Figure 2). Peak bifurcation was variable from one experiment to another (Figure 2B and C), and did not correlate with the presence or absence of the chain-terminating ATP analog CoTP in the binding reactions for EMSA experiments. Although small amounts of ATP from the 5'-end labeling reactions were present in both of the EMSA experiments shown in Figure 2, desalting of oligonucleotides after labeling did not reproducibly affect EMSA data, in either the presence or absence of subsequently added Mg-CoTP (L.Deng and P.D.Gershon, unpublished results). To investigate more fully the possible effects of ATP on VP55–RNA interaction in the EMSA, the 16 tetraU-scanners were incubated with VP55 in the presence or absence of 1 mM Mg-ATP, after first 3'-end labeling the oligonucleotides with the chain terminator [α -³²P]CoTP (Thomson and Gershon, 1995) to inhibit polyadenylation of labeled molecules. Again, bifurcation of the binding peak did not correlate with the presence or absence of ATP (data not shown). Overall, no reproducible, direct effect of nucleoside triphosphates on VP55–RNA interaction was found in any experiment.

Previously, a model was proposed (Figure 9 of Gershon and Moss, 1993b) to account for: (i) the processive elongation of oligoadenylate tails by VP55 to a net length of 30–35 nt followed by an abrupt transition to non-processive polyadenylation; (ii) the non-processive mode of adenylate addition to oligo(A) primers; (iii) the interaction of VP55 with RNA segments 30–40 nt in length; (iv) the necessity for uridylates within the priming RNA for both VP55–RNA interaction and oligo(A) tail formation; and (v) the ability of VP55 to translocate. The proposed model comprised the simplest explanation of the above five observations, namely the initial interaction of VP55 with the 3' OH (for adenylate addition) along with uridylates that are positioned 30–35 nt from the 3' OH, followed by oligo(A) tail elongation in the presence of ATP. VP55 would translocate during elongation of the tail, until a uridylylate recognition site had translocated to the nascent oligo(A). At this point, the polymerase would dissociate, since uridylylate-containing RNA would no longer be in contact with the uridylylate recognition site.

The correlation between the length of uridylylate-containing RNA initially bound by VP55 and the length of the oligo(A) tail added by the enzyme suggested the action of a single RNA-binding site in measuring both the initial substrate and the tail. Although the current data do not contradict the outline of this model, two additional observations should now be accommodated: (i) the presence of dual uridylylate recognition sites; and (ii) two bursts of polyadenylation with tetraU-scanner oligonucleotides 12–16 (Figure 3A), i.e. a 'mini-burst' of 3–12 adenylates which extends the RNA to the 3' side of the 3'-end-proximal uridylylate patch to a net length of ~11–12 nt followed by the 'main' burst which adds a constant length ~35 nt tail to the mini-burst products. With these oligonucleotides, VP55 appears to be able to add up to ~47 adenylates via two oligo(A) tail measuring activities. The formation of mini-burst products through a measuring activity of VP55, as opposed to other possibilities such as a second conformation of either the RNA [e.g. hairpinning of the uridylylate patch with the nascent oligo(A) tail] or the VP55 protein, is supported by the occurrence of mini-burst products with only a subset of the 16 tetraU-scanner oligonucleotides and by the change in mini-burst product size with patch position in a direction opposite to that intuitively expected from oligonucleotide hairpinning.

A model in which translocation is not monotonic might best accommodate the new data (Figure 7). Part 1 of Figure 7 shows the relative positions of the uridylylate recognition sites of VP55. Parts 2–4 show the generation of oligo(A) tails, accounting for the polyadenylation products observed with the tetraU-scanner oligonucleotides (Figure 3A). For tetraU-scanner oligonucleotides 1–9, the uridylylate patch is centered 16–32 nt from the 3' OH. For these oligonucleotides, the ~35 nt (main) polyadenylation burst would initiate while the uridylylate patch is in contact with the 3' OH-distal site. Since there are no additional uridylylate-specific sites upstream of the distal site to which the RNA can translocate in order to effect the main burst of tail elongation, the model shows this burst occurring while the uridylylate patch remains in contact with the distal site, but RNA regions close to the 3'-end translocate with respect to the proximal sites including the associated catalytic site (Figure 7 parts 2–4). This 'tail measuring' activity of VP55 might be accomplished by some kind of conformational change in the protein. For tetraU-scanner oligonucleotides 12–16, in which the uridylylate patch is too close to the oligonucleotide 3' OH for simultaneous interaction of the patch with the distal site and the 3' OH with the catalytic site (Figures 2A and 3A), the tetraU patch presumably interacts with VP55 initially via the 3' OH-proximal sites alone. This interaction would lead to the generation of 'mini-burst' polyadenylation products (Figure 3A). Since the mini-burst products are formed processively (Figure 3B), the RNA presumably remains stably associated with the proximal sites during their formation. The mini-burst products may elongate the oligonucleotides sufficiently to allow the tetraU patch to rebind VP55 via the distal uridylylate contact site while the 3' OH remains in contact with the catalytic site. This would lead to a 'main' burst, as described in the previous paragraph, which would add a 35 nt poly(A) tail to the mini-burst products.

If the distal site is responsible for measuring an ~35 nt

oligo(A) tail, and proximal site B and the associated catalytic site are important for nucleotidyl transfer, what is the role of proximal site A? As suggested above, interaction of uridylates with site A alone may play a role in the generation of mini-burst products. In combination with the distal site, proximal site A may also serve to stabilize RNA–protein interaction. Thus, the NaCl resistance of polyadenylation of the double tetraUmer oligonucleotides (Figure 6C) may result from the simultaneous interaction of uridylates with both these sites at the outset of polyadenylation. However, loss of uridylate contact with either site during adenylate addition presumably causes polyadenylation to cease under high salt conditions. This may account for the observation of oligo(A) tails of less than the expected length of ~35 nt after polyadenylation of the double tetraUmers and the control oligonucleotide rU₃₄ at 150 mM NaCl (Figure 6C). If the distal site predominates during polyadenylation, the reaction could have ceased after the proximal uridylate patch had translocated away from proximal site A (step 2 of Figure 7). If proximal site A predominates, then the observed products (Figure 6C) might correspond to the ‘mini-burst’ products observed with tetraU-scanners 12–16 (Figure 3A). Although a second (main) burst was observed with the tetraU-scanners at low salt (Figure 3A), it did not occur with the double tetraUmers at high salt (Figure 6C). This may be because rebinding of the proximal uridylate patch to the distal site would leave the proximal site devoid of uridylates, rendering the reaction salt sensitive. The polyadenylate products observed with rU₃₄ were ~6–8 nt greater in length than those with the double tetraUmers at elevated salt concentration (Figure 6C). This may have been because the uridylates within rU₃₄ extend 6–8 nt further towards the oligonucleotide 3′ terminus, permitting proximal site A to maintain contact with uridylates during a greater number of adenylate additions. Neither the original model (Gershon and Moss, 1993b) nor that presented in Figure 7 account for all of the properties of VP55. Thus, although Figure 7 accommodates findings from the current study, previous studies have shown that VP55 extends uridylate-free RNA 3′-ends to a constant net length during polyadenylation, as opposed to adding fixed length tails (Gershon and Moss, 1992, 1993b). The action proposed in Figure 7 would be expected to add fixed length tails to model primers such as rU₃₄ and rU₃₄ possessing a pre-formed tail of, say, 15 nt, as opposed to extending tails to a constant net length (Gershon and Moss, 1992). The most enduring aspects of both simple models should be apparent from additional studies.

The possession of dual uridylate-specific RNA binding sites by a PAP would suggest an ‘inchworming’ mechanism of PAP translocation. This is reminiscent of the translocatory mechanism of the DNA-dependent RNA polymerases which occurs at certain sequences within their DNA templates (Krummel and Chamberlin, 1992a,b; Nudler *et al.*, 1994, 1995; Chamberlin, 1995; Wang *et al.*, 1995; Zaychikov *et al.*, 1995). Although the mechanism of nascent transcript extrusion from the DNA-dependent RNA polymerases during transcriptional elongation has not been characterized fully, a role for RNA–protein interactions is indicated by the experimental formation of authentic 1:1 RNA–RNA polymerase complexes in the

absence of template (Altmann *et al.*, 1994; Johnson and Chamberlin, 1994) and the observation that RNA is extruded in a non-monotonic fashion during the ‘inchworm’ translocation of DNA-dependent RNA polymerase along its template (Wang *et al.*, 1995; Gu *et al.*, 1996). RNA–protein transactions occurring during the translocation of the non-templated poly(A) polymerase enzyme might possess features in common with those found in other classes of RNA polymerase.

Materials and methods

Proteins

The purification of VP55 and VP39 has been described previously (Gershon and Moss, 1992, 1993a).

Oligonucleotide synthesis

RNA oligonucleotides and RNA–DNA chimeras were synthesized by solid-phase 2′-O-Fpmp chemistry (Cruachem), according to the manufacturer’s instructions, using an ABI model 392 DNA/RNA synthesizer. DNA–RNA chimera pools were synthesized from phosphoramidite mixtures in single bottles. Prior to pool synthesis, pilot experiments were conducted to calibrate coupling rates for 2′-O-Fpmp riboU versus deoxyriboC phosphoramidites (data not shown). This enabled dC- or dU-containing oligonucleotides to be spiked with riboU at predictable levels. All oligonucleotides were gel purified before use, as described previously (Gershon and Moss, 1992).

Oligonucleotide labeling

Oligonucleotides were 5′-end labeled using T4 polynucleotide kinase, as described previously (Gershon and Moss, 1992). Oligonucleotide 3′-end labeling (CoMP transfer) reactions employed [α -³²P]CoTP (cordycepin triphosphate, DuPont NEN) in combination with either VP55 or VP55 + VP39. Reaction conditions were as described previously (Thomson and Gershon, 1995), except for reaction times, which varied for individual experiments.

Polyadenylation assay and EMSA

The polyadenylation assay and EMSA were performed as described previously (Gershon and Moss, 1993b). Where necessary, RNA was extracted from excised EMSA complexes by overnight soaking in 1% SDS/1 mM EDTA followed by phenol/chloroform extraction and sodium acetate/ethanol precipitation. For EMSA experiments, VP55 and oligonucleotide were used at final concentrations of 20–50 nM and ~500 nM, respectively.

Oligonucleotide partial hydrolysis

Oligonucleotides were partially hydrolyzed by supplementing RNA samples with 1/20 volume of 1 M sodium bicarbonate and heating at 90°C for 10 or 20 min (Whoriskey *et al.*, 1995). Samples were electrophoresed in 9 or 10% polyacrylamide/urea/TBE gels of 0.4 or 0.8 mm thickness, after supplementing the lower buffer tank with 1 M sodium acetate in order to establish an electrolyte gradient (Ausubel *et al.*, 1987). Gels were soaked extensively in 30% methanol/5% glacial acetic acid prior to drying. In control experiments this led to no detectable loss of small oligonucleotide fragments (data not shown).

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